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UNIVERSITY OF ALBERTA

ISOLATION AND TRANSPLANTATION OF CANINE ISLETS

BY

ZILIANG AO

A thesis submitted to the Faculty Of Graduate Studies And Research

in partial fulfilment of the requirements for the degree

of Master of Science

IN

Experimental Surgery

Department of Surgery

Edmonton, Alberta

Fall 1993

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of Canada

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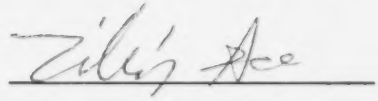

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Date: August 24, 1993

DEDICATION

This text is dedicated to Keuen and our son Peter.

ABSTRACT

Pancreatic islet transplantation is a promising approach for inducing long-term metabolic homeostasis in insulin-dependent diabetic patients. In this study we used canine pancreas as our preclinical model to improve mass isolation of purified viable islets by modifying our digestion, dissociation and purification protocols, and to devise an omental pouch site for islet transplantation.

Canine pancreases were removed by total pancreatectomy and perfused via the ducts with collagenase. Comparisons of purity and yields, and in vivo function of isolated islets were performed among three groups with different islet isolation protocols: manual trituration and teasing (group 1, n=15), automated dissociation modified after Ricordi et al. (group 2, n=26), automated protocol with gentle shaking (group 3, n=13). The islets were purified with Ficoll. The purity of islet preparation was 89.3±1.7% in group 1, 90.8±1.3% in group 2, and 89.2±1.2% in group 3. The number of islets equivalent (IE) to 150 μm in diameter per gram of pancreas in group 1 was 1,374±191, in group 2 was 2,236±328 (versus group 1, p=0.06), and this was further increased in group 3 to 2,515±291 (versus group 1, p<0.02). Normoglycemia was well maintained after islets were autografted into group 1 (n=3) and group 2 (n=7) apancreatic dogs. The K-values at IVGTT were 2.0±0.2% (group 1) and 1.6±0.2 (group 2).

With the same automated dissociation protocol as group 3, purification with Ficoll (Fc, n=13) and Dextran (Dx, n=7) discontinuous density gradient media was compared for islet isolation profile and islet functions in vivo and in vitro. The isolation yield was 2,515±291 IE/gram pancreas in Fc group versus 2,441±584 IE in Dx group (p>0.5) with a islet purity of 89.2±1.2% (Fc) versus 90.7±1.5% (Dx) (P>0.5). Autografts of islets purified with Ficoll (n=7) or Dextran (n=6) could restore normoglycemia, and glucose tolerance was well maintained with K-value of 1.6±0.2% (Fc) versus 1.7±0.2% (Dx) (P>0.5). Glucose

perifusion studies were performed on islets that were purified with Ficoll versus Dextran from the same pancreas. The basal insulin secretion (μU/islet/min) was 0.023±0.007 in Fc group, and 0.043±0.018 in Dx group, and this was increased significantly ($p<0.05$) to 0.068±0.091 and 0.115±0.039 respectively after exposure to high glucose (500 mg/dL). Stimulated insulin response in both groups was biphasic, basal insulin level was restored when glucose returned to 50 mg/dL.

For the omental site studies, islets were isolated using the automated method as in group 3 above. The islets were autografted into a pouch created in the greater omentum (n=12) or into the spleen by venous reflux (n=12). Islet allografts from single donor were implanted into the omental pouch of the dogs that received CsA (n=9) or untreated controls (n=3). The threshold mass of endocrine tissue that consistently reversed diabetes in the omental site was 10 μL/kg, which exceeded by 2.5-fold that required in the splenic site. Normoglycemia was induced and maintained for 2 months in 6 omental and 8 splenic dogs who received more than the threshold mass of islet autografts. All allografts (>10μL islets/kg body weight) in the omental pouch induced initial normoglycemia: grafts of untreated controls failed at 4.2±1.8 days. In one CsA-treated dog the graft failed for technical reasons; normoglycemia persisted in the other 8 for 10, 15, and 21 days, and in 5 instances >30 days. When CsA therapy was stopped at 30 days, normoglycemia persisted for an additional 34±9.5 days.

In summary, compared with a manual method, our modified automated method significantly improved mass isolation of canine islets. Discontinuous density gradients of Dextran were as effective as Ficoll in islet purification. Auto- and allografts of islets in the omental pouch site could restore normoglycemia in diabetic dogs.

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TABLE OF CONTENTS

CHAPTER		PAGE
1	INTRODUCTION	1-25
	REFERENCES	26-37
2	IMPROVEMENTS IN MASS ISOLATION OF CANINE ISLETS BY COMBINATION OF COLLAGENASE PERFUSION AND AUTOMATED DISSOCIATION	38-53
	REFERENCES	54-55
3	A COMPARISON OF FICOLL AND DEXTRAN FOR DISCONTINUOUS DENSITY PURIFICATION OF CANINE ISLETS	56-66
	REFERENCES	67-68
4	SUVIVAL AND FUNCTION OF PURIFIED ISLETS IN THE OMENTAL POUCH SITE OF OUTBRED DOGS	69-86
	REFERENCES	87-89
5	DISCUSSION AND CONCLUSIONS	90-93
	REFERENCES	94

LIST OF TABLES

TABLE		PAGE
2-1	ISLET PURITY AND YIELD BY DIFFERENT PROTOCOLS	46
2-2	DISTRIBUTION OF ISLET SIZE	47
3-1	PROFILE OF ISLET ISOLATION WITH FICOLL AND DEXTRAN	62
4-1	NORMOGLYCEMIA AFTER ISLET AUTOTRANSPLANTATION INTO APANCREATIC RECIPIENTS	73

LIST OF FIGURES

FIGURE PAGE

2-1 SCHEMATIC OF MANUAL AND AUTOMATED ISOLATION PROTOCOLS 48

2-2 FASTING GLUCOSE AFTER AUTOTRANSPLANTATION OF ISLETS INTO THE SPLEEN 49

2-3 PLASMA INSULIN AND GLUCOSE RESPONSE DURING INTRAVENOUS GLUCOSE TOLERANCE TESTS IN DOGS THAT RECEIVED INTRASPLENIC ISLET AUTOGRAFTS 50

3-1 FASTING GLUCOSE AFTER ISLET AUTOTRANSPLANTATION 63

3-2 INSULIN AND GLUCOSE RESPONSES AT IVGTT 64

3-3 INSULIN LEVELS IN THE SPLENIC VEIN AND FEMORAL ARTERY AFTER INTRASPLENIC ISLET AUTOTRANSPLANTATION 65

3-4 GLUCOSE PERIFUSION STIMULATED INSULIN SECRETION BY ISLETS PURIFIED WITH FICOLL OR DEXTRAN 66

4-1 COMPARISON OF FASTING GLUCOSE DURING PROLONGED FOLLOW-UP AFTER TRANSPLANTATION OF ISLETS INTO THE SPLEEN OR OMENTAL POUCH IN APANCREATIC DOGS 82

4-2 COMPARISON OF INSULIN LEVELS IN THE PORTAL VEIN AND THE FEMORAL ARTERY AFTER ISLET AUTOGRAFTS TO THE OMENTAL POUCH 83

4-3 PLASMA INSULIN AND GLUCOSE RESPONSES TO INTRAVENOUS DEXTROSE IN DOGS THAT RECEIVED INTRASPLENIC OR OMENTAL ISLET AUTOGRAFTS 84

4-4 LONG TERM FUNCTION OF PURIFIED SINGLE DONOR CANINE ISLET ALLOGRAFTS IN THE OMENTAL POUCH 85

LIST OF PLATES

PLATE		PAGE
2-1	PHOTOGRAPH SHOWING CANNULATION OF PANCREATIC DUCT	51
2-2	PHOTOGRAPH OF A RECIRCULATING PERFUSION APPARATUS	52
2-3	PHOTOGRAPH OF AN AUTOMATED TISSUE DIGESTION/DISSOCIATION CHAMBER SYSTEM	53
4-1	PHOTOMICROGRAPH OF INTRAOMENTAL ISLET AUTOGRAFT	86

LIST OF ABBREVIATION

Ab	Antibody
Ag	Antigen
ALS	Anti-lymphocyte serum
APC	Antigen presenting cell
CsA	Cyclosporine A
DSPG	15-Deoxyspergualin
FCS	Fetal calf serum
HBSS	Hanks balanced salt solution
IDDM	Insulin Dependent Diabetes Mellitus
IE	Islet equivalent to 150μm size in diameter
IL	Interleukin
IVGTT	Intravenous glucose tolerance test
IRI	Immunoreactive insulin
mAb	Monoclonal antibody
MHC	Major histocompatability complex
PG	Plasma Glucose
RIA	Radioimmunoassay

CHAPTER 1

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is a chronic disease in which the insulin-producing cells (B-cell) in the pancreatic islets are destroyed or altered by mechanisms not well known at present. Since its availability in 1922, insulin injection has been the main treatment for IDDM patients. Unfortunately, despite stringent insulin therapy, half of these patients will suffer complications involving the eyes, the kidneys, and cardiovascular system, which may lead to blindness, renal failure, heart disease, and hypertension. Evidence indicates that the devastating microangiopathic lesions are secondary to the persistent state of hyperglycemia (1), and are due to the inability of our present forms of hormonal replacement therapy to maintain the blood sugar within normal limits at all times. Transplantation of insulin-producing tissue offers the most physiological approach to restoration of normoglycemia and inhibition of progressive neurovascular complications for IDDM. Transplantation of pancreas as a vascularized organ has become popular over the past 20 years, with the results of over 2735 grafts being reported to the International Pancreas Transplant Registry by 1991 (2). Analysis of recent reports of pancreas transplantation reveals that the insulin-requiring state is reversed in 63% of recipients and nephropathy can be prevented in simultaneously transplanted kidneys (3). Although the functional first-year survival of pancreas grafts has improved progressively, clinical application of pancreas transplantation has been limited by its frequent surgical complications with high morbidity and long-term generalized immunosuppression that may cause severe side effects on the patients.

Compared with pancreas transplant, the procedure of islet transplantation is a safer and more simple approach to treatment of IDDM. Furthermore, tissue preservation and

banking is an essential consideration for cadaveric organ transplant to be successful on a large scale. The limit for cold storage of intact pancreas is 48 (4) to 72 hours (5), while islet grafts cryopreserved (frozen-thawed) for periods of up to 6 months have been demonstrated to respond to glucose stimulation in vitro (6,7) and restore normoglycemia in diabetic animals and patients (6-10). The vascularized grafts need long-term generalized immunosuppression that may cause severe side effects in the recipients. Experimental data has suggested that free grafts of islets can be immunomodulated prior to transplantation (11-16) and immunoisolated by micro- or macroencapsulation (17-23) to reduce or eliminate the need for immunosuppression. These advantages have provoked intensive research into islet transplantation for diabetes, and the achievements in islet isolation, preservation, and transplantation in experimental animals during the past 20 years have provided a strong impetus for clinical trials. This introduction will review the significant experimental achievements, particularly in the field of islet isolation and transplantation in large animals.

ISOLATION OF PURIFIED ISLETS

Enzymatic digestion, mechanical dissociation, and density gradient purification are the three key components in current procedure of islet isolation.

1. Intraductal Collagenase Digestion

Routes for collagenase delivery. The first major contribution to islet isolation was made in 1965 by Moskalewski (24) who used collagenase to digest chopped guinea pig pancreas and found the enzyme could result in complete separation of islets from surrounding exocrine tissue. Lacy et al. (25) refined this technique by intraductal distention of the pancreas with Hank's balanced salt solution (HBSS) to disrupt the exocrine tissue prior to mechanical mincing and enzymatic digestion. Although the Lacy technique allowed better results for rodent islet isolation, the approach is not effective when applied to

isolation of islets from large animal and human pancreases. Histological examination of pancreas tissue from various species shows the reason why this approach failed: the pancreas of higher mammals is a markedly more compact and fibrous organ than rodent pancreas, and is relatively undistendable. Development of islet isolation from large animal and human pancreases was delayed for a long time until the changes in the approach to using ductal distention to deliver collagenase (26,27). By using ductal collagenase delivery techniques, it became possible to isolate islets in a large scale first from the dog (26,27,37) and, with further modification, subsequently man (8,28), monkey (29) and pig (30). Now the ductal collagenase delivery technique has been also applied back to rodent islet isolation with marked improvements in both yield and purity (31,32).

The rationale behind the method of intraductal collagenase delivery is to deliver the enzyme into the peri-islet connective tissue matrix through the ductal system and allow the specific enzymatic cleavage of the islets from surrounding exocrine tissue. Ideally, the collagenase should permeate all the exocrine tissue, right up to the peri-islet matrix, but not the islet itself. Studies using intraductal injection of India ink and x-ray contrast material (33,34) have shown that intraductal injection of collagenase results in dispersion of the enzyme throughout the exocrine tissue and, if the injection is continued, the enzyme tends to penetrate into the islets. Because the protease in the impure commercial collagenase preparation may damage the islet integrity by cleaving intra-islet cell-to-cell adhesion (will be discussed later), the penetration of collagenase into islets should be avoided and the injection pressure must be well controlled.

In the intraductal delivery method, collagenase can be delivered by a single injection, termed loading (35), or by a constant perfusion which was initiated by Horaguchi and Merrel (26) and then modified in our laboratory (36-38). Rajotte and Warnock et al. have designed a stainless steel perfusion apparatus with a recirculating constant perfusion pump to

intraductally perfuse the collagenase into the pancreas with precisely controlled temperature and pressure. The possible advantages of perfusion method over loading technique may be that the working pressure and temperature can be precisely controlled and the pancreatic lobules that cannot be distended by loading may be reached by constant perfusion. Comparison studies demonstrated that the perfusion technique improved the recovery of islets from human pancreases when compared with a loading method (39-40).

Collagenase and peri-islet matrix. Despite recent technical improvements, confounding variables in the results of the yield and purity have persisted in our routine islet isolation because of lot-to-lot variation in commercial collagenase. Histological examination of pancreas from various species with immunoperoxidase staining for collagen and electron microscopy (41) demonstrates that the structure of the gland is maintained by a fine network of collagen fibrils that form the peri-islet matrix and acinar septa, and are also distributed in the regions surrounding the blood vessels, ducts, and nerves. The peri-islet matrix, or islet capsule, offers a cleavage plane between islet and exocrine tissue for the specific collagenase digestion, and therefore is of great importance in maintenance of islet integrity during the procedure of isolation. The existence of the islet capsule is of species specificity. The canine islets are completely encapsulated. Human and rat islets are mostly separated from the exocrine by the capsule with some direct islet-to-exocrine cellular contact. In the pig pancreas, the islet capsule is almost absent and the islets are directly apposed to the exocrine by means of cell-to-cell adhesion. These findings may explain why pig islets are notoriously fragile during isolation. Peri-islet matrix is also poorly developed in young pancreases of most species, which may account for the poor yields and purity often reported from juvenile pancreases (35). The study also found that the intra-islet cellular contact almost exclusively depends on cell-to-cell adhesion and is reinforced by desmosomes. It has been documented that the cell-to-cell adhesion in pancreas is susceptible

to proteolytic enzymes and to calcium chelation (42,43).

The commercial Clostridium Histolyticum collagenase used in islet isolation is a mixture of six types of collagenase contaminated by a number of other enzymes, such as neutroprotease, aminopeptidase, clostripain, and phospholipase C (44-47). According to histology findings mentioned above, isolation of intact islets requires selective enzymatic cleavage of islets from exocrine tissue at the peri-islet matrix without cleavage of intra-islet cellular adhesion. A fully purified collagenase seems able to fulfil this purpose. However, some studies (45,48) and our own study (unpublished observation) showed that pure collagenase was not effective in islet isolation, and the isolation ability of various batches of collagenases was found to be correlated best with the content of protease, a major contaminant in commercial collagenase rather than collagenase alone. The failure of pure collagenase in islet isolation may be due to the complicated components in the extracelluar matrix, which consists of not only collagens but also proteoglycans, glycoproteins and elastin (49). An analysis of the roles of collagenase and protease in the extracelluar matrix dissociation for rat islet isolation revealed that total digestion of the matrix collagen component by pure collagenase required the presence of protease activity (50). In extracelluar matrix collagen fibrils are coated with proteoglycans and glycoproteins, which will hinder the access of collagenase to the collagens. Protease can effectively digest proteoglycan and glycoprotein, thereby facilitating the collagenase-induced digestion of collagen-based extracelluar matrix. Protease plays a dual role in islet isolation. On one hand, protease accelerates tissue dissociation by enhancing the degradation of the major components of extracelluar matrix. On the other hand, the enzyme accelerates the process of islet disintegration by cleaving the intra-islet cell-to-cell adhesion. A successful islet isolation requires both collagenolytic and proteolytic activities, but the proteolytic activity

should be limited in order to maintain the integrity of the islets and individual cells. Optimizing the collagenase/protease ratio by blending different type of collagenases has provided better results in dog and human islet isolation in our center (unpublished data).

Additives to digestion solution. Some additives to the collagenase solution are believed helpful in islet isolation, however such additions have not been well studied and their effectiveness remains uncertain. Bovine pancreatic DNAse (51) has been added to the collagenase solution in order to prevent or overcome the formation of gel that is formed by the DNA released from the damaged tissue. Free islets may be stuck to the exocrine by the formed gel. This will affect the results in subsequent purification. Hyaluronidase (52) was added to dissolve hyaluronic acid, another component of extracelluar matrix. Calcium is important for collagenase activity. Gray and McShane (35,45) found that addition of chelating agents such as EDTA into collagenase solution to bind the calcium in the system may inhibit the collagenase activity and stop the procedure of pancreatic tissue digestion. Initial study of human islet isolation (28) suggested that raised calcium concentrations were beneficial, and calcium-containing solutions are still widely used for human islet isolations.

The temperature and end-point of digestion. The activity of commercial collagenase is minimal at temperatures below 4°C. Between 4°C to 43°C the activity increases slowly, increasing in a linear fashion over the range of 30-40°C and peaking at 46°C, above which the enzyme activity decreases, presumedly due to denaturation (45). Although the digestion will progress most rapidly at temperatures higher than physiological, high temperatures above 41°C may result in impaired tissue viability. Temperatures of 37.5-38.5°C are preferred in human and dog islet isolation (36-39), while lower temperatures seem better for pig islets (30). We have noticed that to bring the temperature up to the working point quickly (in 5 minutes) during the initial stage of digestion is crucial for a successful isolation. This will

presumably allow most collagenase to be activated at the same time and dissociate the tissue simultaneously.

Because of the lot-to-lot variation in collagenase activity, the time required for the digestion process is highly variable. An effective approach to determining the end of digestion is the examination of sequential biopsies at microscopy to determine the end-point at which the majority of islets are free from exocrine tissue. The in vitro sequential biopsies for islets were not pratical until the method of rapid islet staining with dithizone was introduced (54,55). Dithizone can specifically stain zinc in insulin of islet B-cells, resulting in a characteristic red stain. This method is now used in many islet laboratories.

Once the end-point has been determined, the action of the collagenase must be terminated. This is usually achieved by a combination of cooling (4°C) and removal of the enzyme by multiple wash. The most widely used washing solution is HBSS, sometimes 2-10% fetal calf serum is added for human and pig islet isolation. It has been suggested that the use of hypertonic organ preservation solution, such as University of Wisconsin solution (UW) or Eurocollins, in the prepurification phase of isolation are beneficial to the tissue viability and purification (35).

2. Mechanical Dissociation

During digestion, enzymatically dissociated islets may remain entrapped by the relatively undigested fibrous capsule and interlobular fibrous tissue, therefore mechanical dissociation is necessary to disrupt the fibrous tissue and release the islets. Choppers (56) or homogenizers (57) have been used for this purpose; however, they were found too traumatic for the islets, resulting in uncontrolled islet damage (28). More gentle forms of dissociation such as teasing, trituration, and shaking appear to maximize islet yields without undue traumatic effects on the tissue. Currently, these methods are widely used and can be classified into a non-automated method and an automated method.

Non-automated method. In this method, the dissociated tissue is gently teased apart with forceps from the undigested fibrous capsule, then is triturated through wide bore needles of progressively smaller size (14, 16, 18 gauge) (28). By using this method we and others have achieved good results in the yields of canine and human islet isolation (8,28,37,38). However, the shearing force from trituration remained too much for the fragile islets, and undigested tissue of over 20% of original pancreas weight persisted, entrapping considerable amounts of islets (38,57).

Automated method. This method was introduced by Ricordi and co-workers (58) by using an automated islet isolator. The essential component of the isolator is a steel-made chamber that is attached to a constant shaker. The chamber is separated by a stainless steel screen and contains several marbles to dissociate the tissue. Pre-warmed collagenase solution can be recirculated by a constant pump in the closed chamber system where pancreas is continuously digested and dissociated. The advantages of the automated method over the non-automated technique include less traumatic shearing force to the islets, better tissue dissociation and digestion, and reduced chance for contamination with minimal human intervention. We have modified the automated isolator by using a glass water jacket chamber that allows a direct observation of the extent of tissue dissociation, which is an important index in determining the digestion end-point. Our comparison studies (38) have demonstrated that the automated method with gentle shaking techniques provided better results in isolation yields and islet size distribution when compared with the non-automated method.

3. Islet Purification

Following the dissociation, the islets must be purified. Early studies (59,60) suggested that purification steps could be eliminated to prevent the loss of islet tissue. Although this

approach provided adequate quantities of islets to reverse diabetes in autotransplantations,wide-spread success has been denied. Several disadvantages of the non-purification approach have been described in later studies. Unpurified grafts may cause severe postoperative complications such as portal hypertension, disseminated intravascular coagulation, and sepsis (61-63). Protease released by the contaminating exocrine cells could cause autodigestion (64). Contaminating exocrine and lymphatic tissue enhance the immunogenicity and accelerate the rejection of islet allografts (64,65). The above findings suggest that purification of islets is beneficial for islet transplantation.

Density gradient purification. Numerous techniques have been developed for purification of collagenase-dissociated islets, including filtration (66), centrifugal elutriation (67), differential sedimentation at unit gravity (69), fluorescent-activated cell sorting (70), and density gradient centrifugation. Density gradient centrifugation, first introduced by Ballinger and Lacy (71), is the most widely used method for large-scale islet purification. This density dependent or isopycnic cell separation is achieved by centrifugation for sufficient time to force cells to arrive at the location in the gradient where their densities are equal to that of the gradient. Obviously, the density difference between exocrine and islets will determine the efficiency of cell separation, or in other words overlapped densities for islet and exocrine tissue will result in incomplete cell separation.

Studies have found that the density of exocrine tissue is more variable and more sensitive to changed conditions than that of islets, therefore, is the primary cause for variation in success of islet purification. The peak density of exocrine tissue ranges from 1.059 to greater than 1.074 g/mL and varies markedly between pancreases, while the peak density of islets is 1.059 g/mL without much individual variation (72). The density of acinar cells is more dependent on their secretory status. Acinar cells contain large granules (up to

1.5 μm), a majority of which will be discharged in response to stimulation. In contrast, the insulin granules in B-cells are relatively small (0.4 μm) and only a maximum 10% of thestored insulin are released at stimulation (73-75). The exocrine densities may be altered by numerous factors, which act either by causing exocrine degranulation or by leading cellular swelling or edema. Exocrine degranulation may result from the use of vasopressor drugs, hypertension and hypoxia state of the donor, prolonged warm and cold organ ischemia, or mechanical and enzymatic cellular damage during isolation procedure. The cellular swelling of exocrine tissue may be caused by reduced cell viability or exposure to hypoosmolar environment during isolation. Degranulation and cellular swelling will reduce the density of exocrine tissue and subsequently ruin the islet purification with density gradient centrifugation. Based on these considerations, many approaches have been suggested in different isolation stages to prevent or overcome exocrine degranulation and cellular swelling. They include minimized warm and cold ischemia time at pancreas procurement, optimized digestion time and working temperature, use of gentle dissociation techniques, and use of hyperosmolar tissue preservation solution such as UW solution in organ flush, tissue washing, and storage prior to purification (76,77).

Density gradient media. Many density gradient media have been used for islet purification, such as percoll (78), Ficoll (79), sucrose (25), dextran (80), metrizamide (81), and bovine serum albumin (BSA) (82). The three more commonly used gradient media for human and large animals at present are Ficoll, BSA, and dextran, though their relative pros and cons for islet purification have been a source of debate in the literature. Ficoll, a copolymer of sucrose and epichlorhydrin with an average molecular weight of 400,000, has been most-widely used with the advantages of formation of clear-cut density layers, ability to be autoclaved, and derivation from a non-animal source. Its disadvantages include enhanced cell aggregation at centrifugation (83), high osmotic activity by binding water (84),

and reported dialysable cytotoxic effect to islets (85,86). BSA has the advantages of general protective effects on cells and reduced cell aggregation (83); however, its animal origin increases the chance of contamination which greatly limits its clinical application. Dextran is a polysaccharide with a molecular weight of 70,000 and clinically used as a plasma substitute. Dextran is less expensive than the other two media discussed, less toxic to the tissue than Ficoll (85,86), and is autoclaveable. Our studies (see Chapter 3) and others (87-89) showed that dextran density gradient purification provided better or similar results in the yield, purity, and viability of islets isolated from rodent and dog pancreases when compared with Ficoll.

Interestingly, recent developments in islet isolation have shown that the solvent for the density media is even more important than the media itself in improving islet purification. Eurocollins and UW solution are the two widely used solvents for density media, which are believed to prevent cellular swelling, exocrine degranulation, and to offer other cell protection. Studies have demonstrated Eurocollins-Ficoll or more recently, UW-Ficoll improved the yields and purity of islets isolated from dog, pig, and human pancreases (90-93).

Top versus bottom loading, discontinuous versus continuous density gradient. A further area of relevance to the islet purification is the top versus bottom loading and discontinuous versus continuous gradient technique. The top loading method has the advantage of keeping the digested tissue in physiological media for the maximum possible time. However, it increases cell aggregation (90) and compromises islet separation because a large volume of heavier exocrine tissue mass travels from the top to the bottom at centrifugation which may drag the islets to the pellet. For these reasons, bottom loading has been preferred by most centers. Compared with discontinuous gradient purification, the continuous method may be superior. This causes less cell aggregation at the interfaces of

individual density gradients, thereby allowing better cell separation. Furthermore, continuous gradients offer more options for choosing the final graft purity. The major limitation for its large scale application is the technical difficulty in constructing a large continuous gradient. Therefore, discontinuous gradient purification still remains widely used for large scale islet isolation.

Based on the considerations discussed above, a new islet processor COBE 2991, has recently been developed for large-scale islet isolation. By using a ring-type plastic centrifugal bag, the COBE 2991 offers many advantages. These include shortened operation time, ability to process a large volume of tissue in a closed sterile system, construction of large-scale continuous gradients, and capability of unloading the purified tissue without deceleration, therefore minimizing the swirl of separated tissue layers. Initial trials for COBE 2991 in our center and others (77) have proved that COBE 2991 is effective in mass purification of human and canine islets.

ISLET TRANSPLANTATION

In 1893 Williams made the first effort to transplant sheep pancreatic fragments into a diabetic patient. Eighty years later Ballinger and Lacy (71) reported the first successful autotransplantation of isolated islets in rats. Since then, with the advances in islet isolation, preservation and antirejection therapy, great achievements have been made in transplantation of islet auto-, allo-, and xenografts in animals and humans. The effectiveness of islet transplantation for diabetes may be affected by multiple factors, including the quantity of islets grafted, viability of the islets, site and method of implantation, revascularization of the grafts, efficiency of antirejection agents and the influence of immunosuppressive drugs on the graft and the recipient. In the following the relevances to the islet transplantation will be discussed.

1. Threshold Mass of Islet Grafts

The determination of threshold mass of islet grafts that are needed to reverse diabetes is critical in predicting the functional outcomes of the islet autografts. Furthermore, due to a lack of specific methods to monitor the rejection of islet allo- or xenografts, the determination of threshold mass of islet grafts is of particularly significance in distinguishing whether the graft failure is due to immune rejection or due to insufficient quantities of islets provided in the grafts. In dogs, 90% subtotal pancreatectomy results in overt diabetes (95). In human 95% pancreatectomy can induce diabetes (96). It is estimated that 10% of the original islet mass will prevent diabetes (103), however the threshold mass of islet autografts are likely higher because of reduced islet viability, altered blood perfusion after neovascularization of the grafts in the heterotopic site and denervation of the islets (97). Progressive improvements in mass isolation of highly purified islets has made it possible to define the threshold graft mass for islet transplantation in large animals by implanting different quantities of islet autografts into pancreatectomized recipients. Our studies have shown that the threshold graft mass is 4-5 μL/kg body weight (about 2,500-3,000 islets equivalent (IE) to 150 μm in diameter) for dog islet autografts in splenic site (98,99,102). Munn and Kaufman reported similar results in their studies and found that the threshold islet mass for intraportal autografts is the same as for intrasplenic autografts (100,101). The omental pouch site needs more islets to reverse diabetes and the threshold graft mass is 10 μL/kg (about 6,000 IE/kg), presumably due to delayed and/or inferior revascularization of the islet autografts (99). The implantation methods also affect the mass of islets required. Warnock et al. found that with similar quantities of islets, those grafted into the spleen by venous reflux functioned better than those by intrapulp injection (102). The definition of threshold islet mass in dogs suggests that a 70 kg pancreatectomized human recipient may

require at least 210,000 IE to reverse diabetes. This estimation compares favorably with the results of human isletautotransplantation, but contrasts with the general experience of islet transplantation in diabetic patients, in which an endocrine mass of 10,000 IE/kg was found to be necessary to alleviate insulin independence (8,10,103). This discrepancy may be ascribed to the adverse influence of immunosuppressive drugs (104,105), microvascular changes (106), and the insulin resistance resulting from the chronic diabetic state (107).

2. Transplantation Sites

An ideal transplantation site for islets would be one in which the surgical hazards and postoperative complications are minimized to the patient, insular hormones are delivered directly into the portal system, sufficient blood perfusion is provided to facilitate the islet engraftment, islet grafts can be retrieved for biopsy purpose. Many different sites have been used for islet transplantation in rodents, including the liver, spleen, renal subcapsule, peritoneal cavity, muscle, brain and testis. However, the most successes for islet transplantation in large animals and humans have been limited to the splenic and hepatic sites. Early studies with autografts of impure pancreatic microfragments showed that the spleen could accommodate large quantity of graft tissue and normoglycemia was restored by intrasplenic islets (108-110). As mentioned before, the intrasplenic islet grafts by venous reflux provided better metabolic function than those by direct parenchymal injection into the splenic pulp. These findings have been confirmed by our laboratory and others with highly purified islets (102,111). Intrahepatic grafts of pancreatic microfragments by intraportal injection were not as successful as their splenic counterparts with some severe complications such as elevation of serum liver enzymes, disseminated intravascular coagulation and portal hypertension (61,112,113). Recent advances of islet isolation in large scale have made it possible to transplant highly purified islets into the portal vein in dogs without evidence of

the complications discussed above (114). Warnock et al. (103) found that the intrasplenic islet grafts had significant longer survival than intraportal grafts after up to 4 years follow-up. Eighty percent of the intraportal autografts failed within the first 10 months and none of them could maintain euglycemia by 2 years. By contrast, all of the intrasplenic grafts functioned for at least 1 year, 80% of them remained normoglycemia for 2 years, and some continued their functions for up to 4 years. Scharp (115) et al. in their dog study also found that the intrasplenic islets survived longer than intraportal islets after 1-year follow-up. The reasons for the discrepancy are not clear. Detailed morphological study with intraportal islet autografts in rodents showed single islet cells outside the portal area, directly apposed to hepatocytes (116). Many individual B cells were also found intercalated between hepatocytes following intraportal islet autotransplant in dogs (51). These findings suggest the disruption of islet structure in intrahepatic grafts and this may be relevant to the accelerated failure of intraportal islets. Another hypothesis is that the spleen might produce some trophic factors that enhance the islet engraftment (103).

Renal subcapsule is not a suitable implant site for large animal and human islets, although it has been widely used in rodents (117). Islet engraftment is difficult when relatively large volume of canine islets implanted into the limited space of renal subcapsule, often with uncontrollable haematoma (unpublished observation).

The intraperitoneal site has recently attracted investigators' attention because it may be an ideal site for engraftment of microencapsulated islets. Intraperitoneal cavity has been used for free islets or microencapsulated islets in rodents (118,119) and encapsulated islets in dogs (20). Use of omental site has been reported for isolated islets in rodents (120) and miniature swine (121). Altman reported infusion of islets into an epiploic artery in human subjects (122). We have designed an omental pouch (see Chapter 4) by suturing and folding the leaves of greater omentum together for islet transplantation in dogs (99). The

intraomental islet autografts had prolonged survival with maintained normoglycemia in 3 months follow-up, despite requirement for higher threshold islet mass that was twice as much as for splenic site, and relatively impaired metabolic function compared with in the splenic site. It is interesting that the islet allografts in the omental pouch had longer survival with CsA immunosuppression compared with intrasplenic allografts, and the graft function persisted for more than 30 days (up to 50 days) after cessation of CsA. Several factors may account for the allograft results compared between the omental and splenic sites. First, higher activities of cell-mediated immune effector responses in the spleen may accelerate the rejection of intrasplenic islets. Second, a slower clearance of lipophilic CsA from the fatty omentum locally enhances the CsA concentration in the omental tissue. Finally, presentation of donor antigens via lymphatics in the omental route might differ from that by direct intravascular injection in the spleen.

The omental site offers several practical and theoretical advantages. The creation of the pouch is technically simple, grafts in the omental pouch can be retrieved for biopsy, the pouch may be suitable for transplantation of microencapsulated islets in large animals and humans. Drugs such as growth factors (124) or immunosuppressants (125,126) could be applied directly to the pouch to enhance islet engraftment or to obviate or minimize the use of generalized immunosuppression.

3. Metabolic Control After Islet Transplantation

The central rationale behind islet transplantation is to prevent or stabilize the chronic microvascular complications of IDDM by maintaining precisely controlled glucose metabolism. Metabolic studies demonstrated that the dogs with intrasplenic autografts of pancreatic microfragments (108-110) or purified islets (99,100,103,114,115) could maintain fasting normoglycemia at long-term follow-up, however, their K values at IVGTT were reduced with blunted insulin release compared with normal controls. The impaired glucose

tolerance may be due to the reduced islet mass. Scharp et al. (115) found a significant correlation of K values with the quantities of the islets that were autografted in the dog. Further studies of long-term islet-autografted dogs showed that the defect in glucose tolerance was due to reduced effectiveness in the ability to enhance its own disposal (127). Mixed meal oral glucose tolerance tests (MMOGTT) (115) have demonstrated significantly elevated glucose levels with high insulin concentration in islet autografted dogs. This suggests that the insulin resistance might be an additional cause for the impaired glucose tolerance. The reasons for insulin resistance in these islet autografed animals are at present only speculative. Some investigators found that the insulin sensitivity in islet autografted dogs was well preserved (127), therefore insulin resisistance in islet autogafted recipients may due to other factors. First, IVGTTs or MMOGTTs in some canine autotransplant studies demonstrated a reduced first-phase insulin release with a delayed return to the baseline insulin level compared with normal dogs (115,129). This altered pattern of insulin release may partially account for the resistance. Second, more insulin secreted by the heterotopic islets, such as intrahepatic islets, may by-pass the liver and be directly released into the periphery. This might induce insulin resistance, although transposing venous drainage from splenic site to systemic circulation showed no significant difference in performance of IVGTT and MMOGTT before and after the shunt (130). Furthermore, insulin deficiency by itself may also cause insulin resistance (128).

The counterregulatory capacity after islet transplantation have rarely been studied. The only published data by Scharp et al. (115) reveals that glucagon secretion by islet autografts at IVGTT did not differ from the controls. However, when stimulated more powerfully by a protein meal the glucagon concentration rose higher in transplanted dogs than in normal controls. Since glucagon is a well-known diabetogenic hormone, its deranged

secretion pattern might contribute to the failure of long-term islet autografts. This issue deserves more detailed studies, due to its possible clinical relevance.

More recent studies have focused on the effects of denervation of islet grafts on their metabolic function. Following oral mixed meal feeding (129), the insulin levels in the venous effluent from spleen transplant site were characterized by absence of vagal-mediated initial "burst". This results in an exaggerated rise in plasma glucose, indicating that denervation of transplanted islets causes impaired glycemic control. The response to moderate exercise in islet autografted dogs (131) was characterized by appropriate suppression of plasma glucose levels, but the post-exercise return to basal levels was delayed compared with controls, suggesting an abnormal response to circulating catecholamine (103) which might also result from the graft denervation.

Theoretically, islet transplantation could prevent or reverse diabetic retinopathy (135), neuropathy (136), and renal complications (132-134) in rodents. However, there is little data of the effects of islet transplantation on chronic complications in large animals. Such study has been limited in large animals by the prohibitive expense of long-term maintenance and delayed graft failure after long-term follow-up. These studies need to be performed in future investigation.

4. Anti-rejection Treatments for Allo- and Xeno-grafts

Immunorejection has been the major obstacle for wide-spread application of islet transplantation in IDDM patients. Among 124 human adult islet allografts since 1974, only 30% of the cases in 1990 and less than 2% of cases between 1974 and 1989 were off insulin for a short time (>1 week) (137). However, the demonstration of long-term insulin independence (>2 years) after islet allotransplant in a IDDM patient (9) gives rise to a hope to the achievement of long-term survival of islet allografts. The remarkable accomplishments

in the area of anti-rejection therapy, tissue immunomodulation and artificial immunoisolation in animal experiments have provided a wealth of new basic information for transplantationimmunology, and undoubtedly will accelerate the development of islet transplantation for IDDM patients.

Recipient Immunosuppression. Generalized immunosuppression with immunosuppressive drugs and antilymphocyte antibodies peri- and post-transplantation have provided an effective approach to prolongation of islet allograft survival.

Cyclosporine A (CsA) has attained a deserved reputation as one of the most effective immunosuppressants in experimental and clinical islet transplantation (8,89,138,139). Initial attempts at islet transplantation utilizing oral administration of CsA failed to extend allograft survival(140), however intramuscular injection of CsA in combination with dose adjustment guided by serum or blood CsA levels, a method to increase the bioavailability of the drugs, has achieved reproducible prolongation of islet allograft survival in large animals(89,138,139). Studies found that the initial depth of immunosuppression in CsA monotherapy is critical to prevent allograft rejection. Insufficient initial CsA level, (<600 ng/mL blood) resulted in early graft failure in dogs(89). Many investigators have reported the detrimental effects of CsA on islet graft function(89,138). Alejandro et al. (141) found CsA could cause irreversible impairment in islet secretory responses.

Azathioprine and **prednisone**, in contrast to CsA, failed to achieve widespread success in prolongation of islet allograft survival in large animals and have been found deleterious to islets(109.139,140,143). However, when they were used in combination with CsA or/and antilymphocyte serum, allograft survival was improved in human and dogs(89,143).

There are very limited reports on the new immunosuppressive drugs, such as FK506, rapamycin and 15-Deoxyspergualin (DSPG) in islet cell transplantation, although their effectiveness have been detailed in organ transplantation.

FK506, known to block transcription of mRNA for IL-2, has been reported effective in prolongation of islet xenograft survival (human to rat) (144), however, as other immunosuppressants, it has been found detrimental to islet function(141).

Rapamycin. A recent study in our laboratory has demonstrated that rapamycin introduced marked prolongation of islet allograft survival in mice with a short course of administration (7 days) (145). It has been documented that rapamycin and CsA are synergistic in blocking lymphocytokine signal transduction(169). Kneteman et al. (unpublished data) found combination of low-dose rapamycin and CsA extended canine islet allograft survival without obvious toxic effects on the grafts compared with single agent control groups.

DSPG (15-Deoxyspergualin) was also shown effective in prolongation of islet xenograft survival (hamster to rat). However three of ten recipients died while normoglycemic (146), suggesting a severe toxicity to the recipients at the dosage.

Antilymphocyte antibodies. Many kinds of these Abs have been used as anti-rejection therapy for islet allo- or xeno-grafts, including polyclonal Abs such as antilymphocyte serum (ALS) and monoclonal anti-T-cell Abs such as OKT3 (anti-CD3), GK1.5 (anti-CD4). Administration of these Abs with a single dose can temporarily delete or paralyse or modulate (150) the donor-Ag-specific T-cells in the recipient to prevent the onset of graft rejection. Therefore, these Abs are widely used in the initial stage of islet allo- or xenotransplantation in combination with immunosuppressive drugs. ALS combined with CsA based anti-rejection therapy has prolonged allograft survival in human islet transplantation (8,9). Peritransplant administration of GK1.5 mAb resulted in long-term survival of islet allograft in untreated rodents (147-149). Anti canine T-Cell mAb (5G2) in combination with low dose CsA prolonged the survival of intrahepatic islet allografts (151).

With the introduction of new immunosuppressive agents and better understanding of the mechanisms of rejection, continued improvements have been made in graft and recipient survival. However, current systemic administration of the immunosuppressants prevents transplant rejection at the cost of impaired tissue or organ function due to their toxicity. Optimized combination of synergistic immunosuppressive agents may enhance their pharmacological effects and minimize their cellular toxicity. Furthermore, local immunosuppression may be another strategy for anti-rejection therapy. The rationale behind local immunosuppression is to prevent rejection by suppressing only those immunological events occurring at the graft site without general adverse consequences of generalized immunosuppression. The major obstacle for its application in kidney, liver, or heart transplantation is the difficulty in establishing a pharmacokinetic advantage of regional drug delivery which is limited by high blood flow or drug clearance function of the organs(125). Local immunosuppression may be more suitable for islet grafts, which can be implanted into a site, such as omentum, where drugs can be locally delivered and establish high tissue concentration. Little data on local immunosuppression so far has been published in islet transplantation. A recent study showed that local delivery of ALS at the transplant site provided marked prolongation of islet xenografts (rat to mouse) (126). Investigations in the field of local immunosuppression need to be further detailed.

Graft immunomodulation prior to transplant. Long-term survival of islet allo- or xeno-grafts have been achieved following pretreatment of donor tissue to reduce tissue immunogenicity. According to Snell's "passenger lymphocyte" theory, the lymphocytes (presumedly dendritic cells and macrophages) in the donor tissue provide the primary trigger mechanism for immune rejection. Many studies have demonstrated that selective depletion of these passenger lymphocytes prolong allo- or xeno-graft survival.

Based on Opelz and Terasaki's observations that low temperature culture at 22°C inactivated the ability of lymphocytes in mixed lymphocyte reaction, Lacy et al. demonstrated low temperature culture (24°C) of donor rat islets combined with single injection of ALS allowed prolonged survival of allografts (153). Then these results have extended to xenografts in rodents (154,155).

Tissue culture in high oxygen could also reduce graft immunogenicity (162). Lacy and coworkers found that the rat to mouse islet xenografts had a prolonged survival following pretreatment with 7-day culture at high oxygen (95% air: 5% CO_2) (154,155). Our laboratory observed that prolonged in vitro low temperature culture (7-day, 95% air and 5% CO_2 at 22°C) resulted in extended survival of canine allografts with low dose CsA (156).

UV-B light can suppress DNA synthesis and at high dose is toxic to rapidly dividing cells, such as lymphocytes, therefore UV-B is widely used on donor tissue to induce graft immunomodulation or down-regulation of the host immune responses. Evidence has shown that UV-B irradiation of islets could induce tolerance or prolonged survival of allo- or xenografts in rodents (157). Unresponsiveness to islet allografts was achieved by recipient treatment with UV-B irradiated donor specific blood transfusion (158). Intrathymic injection of UV-B irradiated spleen cells combined with peritransplant ALS injection induced permanent acceptance of islet allografts in rats(157). In contrast, UV-B irradiation is relatively ineffective in large animals. UV-B irradiation of allogeneic islet in combination with recipient pretreatment of CsA resulted in only short term normoglycemia (5-9 days) in 3 of 9 ABO compatible Cynomolgus monkeys (159).

Discovery of lymphocyte-specific surface epitopes made it possible to deplete passenger lymphocytes by selective antibody pretreatment to the donor tissue. Pretreatment with donor specific MHC Class II Abs on freshly isolated islets resulted in the selective elimination of MHC Class II positive lymphocytes from the donor islet tissue, and prolonged

the survival of the islet allografts in mice (160,161). It has been suggested that MHC Class I Ags on the surfaces of all parenchymal tissue cells, including islet cells, also play important roles in immunorejection by interaction with host cytotoxic T-cells in effector mechanisms. Faustman et al. (16) used highly purified MHC Class I Ab fragments (F(ab)) to pretreat donor islets. The fragments masked the class I antigens and prolonged the function of human C-peptide secretion for up to 200 days after transplantation (human to mouse) without immunosuppression.

Immunoisolation of islet grafts. Immunoisolation of the islets by bioartificial micro- or macrocapsules (bioartificial pancreas) is an attractive solution to immune rejection. Generally, the mechanism of immunoisolation is that the islet grafts are separated from the host immune system by a selectively permeable artificial membrane that permits passage of low molecular weight substances such as nutrients and insulin, but not of the cytotoxic cells or high molecular weight immunoglobulins. Based on the above principle, many membranes formulated with different materials have been developed for micro- and macro-encapsulation of islets.

Microencapsulation. The most widely used microcapsules for islets are alginate-based microcapsules. More than a decade ago, Lim and Sun (17) first described the islet microencapsulation procedure with alginate membrane and reported the reversal of diabetes in rats by microencapsulated islet allografts. Since then similar successes have been extended to microencapsulated islet xenografts in rodents (21,164) and more recently allografts in spontaneous diabetic dogs (20). Despite these encouraging results, researchers in this field have been frustrated by the inconsistency of results, some demonstrating long term success, while others report early graft failure caused by intensive cellular reaction and fibrous overgrowth around the capsulated islets within a short period of time following transplant. The activated lymphocytes may release some toxic factors to the islets and the fibrosis around

the capsule will interrupt the metabolic exchange of the encapsulated islets. The reasons for the overactive cellular or fibrous proliferation is not very clear. In some cases it seems due to poor biocompatibility of the capsules. Soon-Shiong found that high mannuronic acid (M) components in the alginate-based membrane is a potent stimulator for fibrous response, and reduction of the M content in membrane formulation can obviate the fibrous overgrowth (20). In other cases it appears due to the release of donor Ags from the capsulated islet tissue. Weber and others (164,165) observed that empty alginate-lysine microcapsules implanted intraperitoneally in mice elicited no cellular reaction in a 1-6 months follow-up, while microencapsules containing xenogenic islets could provoke intense cellular responses particularly in NOD mice. Treatment of the donor islets with UV-B irradiation followed by encapsulation prolonged the xenografts (rat to mice) survival with only a mild cellular reaction (166). Based on the evidence that UV-B irradiation can impair the production of IL-1 and IL-2 by lymphocytes, Weber (164) postulated that lymphokines such as IL-1 and IL-2 (molecular weight 15,000-17,000 kd) as well as other soluble Ags of donor origin, such as Class I Ag (44,000 kd) and Class II Ag (34,000 kd), might shed and be released from the encapsulated islet tissue contaminated with lymphoid cells. These Ag molecules function as important factors in provoking host immune responses to the encapsulated allo- or xenogenic islets. This postulation has been supported by the evidence that large molecules such as haemoglobin (64,500 kd) (167) and albumin (68,000) (168) could be rapidly released from alginate-based microcapsules.

Macroencapsulation. Another method for islet immunoisolation is macroencapsulation in which a mass of islet grafts is macroencapsulated in a selectively permeable membrane diffusion chamber or device. These membrane diffusion chambers or devices in different shapes can be implanted extra- or intravascularly as a bioartificial (or hybrid) pancreas. By using macroencapsulation, long-term xenograft survivals have been

obtained in streptozotocin-induced diabetic rats (22,23). The major limitations of this approach include poor biocompatibility, limited capacity for large quantity of islets in large animals, and intravascular embolism.

The mechanisms affecting the biocompatibility, mechanical and chemical integrity of the artificial micro- and macrocapsules remain to be further studied, and indeed these immunoisolation approaches in combination with islet immunomodulation offer the promise to apply islet transplantation into IDDM patients.

SUMMARY

Despite remarkable advances in islet transplantation during the past 20 years, improvement of large-scale islet isolation, development of new transplant site for islets, and prevention of immune rejection remain as challenges for investigators in this field. In the studies presented in following chapters we have used dogs as our preclinical model to improve the digestion, dissociation and purification protocols for large-scale islet isolation, to develop and evaluate a new omental site for islet transplantation, and to investigate the survival and functions of islet allografts in this site with CsA therapy.

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CHAPTER 2

IMPROVEMENTS IN MASS ISOLATION OF CANINE ISLETS BY COMBINATION OF COLLAGENASE PERFUSION AND AUTOMATED DISSOCIATION

INTRODUCTION

Insufficient yield of purified islets from pancreas has been a main barrier to islet transplantation for insulin-dependent diabetes. Since the collagenase method was introduced for mass isolation of islets (1), various techniques have been developed to improve the mass isolation of islets from animal models and humans. They included ductal loading or perfusion of collagenase (2-4) to digest the fibrous connective tissue stroma that binds islets to the exocrine tissue, and manual (5) or automated (6) mechanical dissociation of the digested tissue. However, an optimal combination of these techniques still remains to be defined to maximize the yield of highly purified viable islets. Since Ricordi et al. introduced an automated method for islet isolation, this method has been successful in islet isolation from pancreases of humans, pigs, and dogs (6-9). Warnock et al. improved the mass isolation of canine islets by combining the automated method with ductal collagenase perfusion, but the increase in the yield and size of isolated islets failed to reach statistical significance when compared with the manual method (10).

The aims of the present study were to use canine pancreas as our preclinical model to improve mass islet isolation by combination of ductal collagenase perfusion with modified

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dissociation protocols. The manual versus automated dissociation method was compared by evaluating the purity, size, yield, and in vivo function of isolated islets.

MATERIALS AND METHODS

Experimental design

Figure 2-1 summarizes the manual and automated dissociation procedures in which three protocols were completed in succession as follows: manual dissociation (group 1, n=15); automated dissociation (group 2, n=26); and automated method with gentle shaking technique (group 3, n=13).

Isolation of Islets

Fifty-four consecutive pancreases of adult mongrel dogs (body weight 18-25 kg) were mobilized with all major vascular connections maintained until excision of the gland. The right and left main branches of the pancreatic duct were cannulated in situ using PE90 cannulae, and an extra cannulation was made into the left duct via a cut-down 8 cm from the distal end (plate 2-1). The pancreas was weighed, cooled in chilled Hank's balanced salt solution (HBSS), and injected with 50 mL collagenase (type V, Sigma, St. Louis, MO, 2 mg/mL in HBSS) into each cannula, which was then attached to a recirculating perfusion apparatus (4) and perfused with collagenase (2 mg/mL, 4°C) (plate 2-2). At 10 minutes, the temperature of the perfusate was increased gradually to 37°C or 30°C at a rate of 3°C/min. The pressure of perfusion was monitored and adjusted to 300 mmHg.

Group 1 pancreas, as we reported previously (10), was continuously perfused at 37°C until the gland became mucoid, lost its distended appearance, and the main duct was visible through the softened parenchyma. The digested tissue was immersed in chilled HBSS with 5% fetal calf serum (FCS), gently dissociated with forceps, washed with and resuspended in

chilled HBSS with FCS, and then mechanically dispersed by aspirations and injections through graded needles (14 gauge x3, 16 gauge x3, 18 gauge x2). The end of dissociation was determined by examining the tissue through a stereomicroscope for the percentage of free islets (80-90% free at end point). Group 2 and group 3 pancreases were perfused until the perfusate temperature reached 30°C, and then immediately transferred to an automated shaking device modified after Ricordi et al (plate 2-3). (6) for continuous digestion and dissociation. The shaking chamber was made of a glass water jacket consisting of two portions separated by a stainless steel screen (opening size 400 μm) and connecting to a glass heating/cooling column system. The collagenase (37°C) filled and recirculated in the closed chamber system. In group 2, continuous shaking of the chamber was started at 10 minutes after loading the pancreas and this was maintained until the end of dissociation. In group 3, a gentle shaking technique was used. This is defined by shaking which did not start until 20 minutes and was maintained intermittently for periods of 20 seconds at one minute intervals while sampling. The end point of both dissociations were determined by using the same method as in manual group. At dilution stage, the chamber still was kept warm allowing continuous digestion of the undigested tissue, while the collection part of the system was kept cold to inactivate the collagenase. Digested tissue was washed x3 by cold HBSS, combined, and resuspended in chilled Medium 199 in a ratio of 0.6 g tissue to 1 mL Medium 199.

Purification

The digested tissue was purified with Ficoll discontinuous density gradient (4) by following procedures (4). Ficoll (MW 400,000, Sigma Chemical Co., St. Louis, MO) was adjusted to pH 7 before autoclave sterilization. Four millilitre aliquots of tissue suspension were vortexed with 21 mL of Ficoll (density 1.100) contained in 50 mL conical tubes, and 5 mL of Ficoll (density 1.085, 1.075, 1.045) were overlaid successively. The tissue was then

centrifuged at 1500 rpm for 25 minutes at 22°C. The purified islets were harvested from the interfaces of the upper layers of Ficoll and washed with Medium 199 x3.

Quantification

Islet mass was quantified and purity assessed in accordance with criteria established at the 1989 International Workshop on Islet Assessment (11). Briefly, aliquots from the islet suspension were incubated with Dithizone and examined microscopically with the aid of an optical graticule. The number of islets in the diameter ranges (60-99, 100-149, 150-199, 200-249, >250 μm) was counted by two individual investigators and the total in each range was converted to the number of islet equivalents (IE) to 150 μm in diameter. The purity for insulin-containing islets was estimated by comparing the relative quantities of Dithizone stained and unstained tissue at microscopy.

Autotransplantation

To assess viability in vivo, the islets from group 1 (n=3, 2717±340 IE/kg) and group 2 (n=7, 3679±623 IE/kg) were autotransplanted into the spleen by venous reflux. The fasting plasma glucose (PG) was measured three times a week for 2 months with a glucose oxidase method (Type II glucose analyzer, Beckman, Fullerton, CA). Intravenous glucose tolerance tests (IVGTT) were performed at 8 weeks postimplant. K values (decline of PG, %/min) at IVGTT were calculated from PG levels at 5, 10, 15, and 30 minutes after injection of Dextrose (0.5 g/kg) (12). Plasma was collected simultaneously for assay of immunoreactive insulin (IRI) using double-antibody RIA with human insulin standards (Pharmacia, Uppsala, Sweden) (13). At the conclusion of follow-up, general aesthesia and laparotomy were performed on autograft recipients, and the spleen bearing the islet graft was removed, and then the animals were returned to the vivarium where PG was monitored daily for at least 3 days.

Data Analysis

All results were expressed as mean±SE. Where appropiate, the two-tailed Students' t-test for unpaired data were used to determine the significance of differences between parameters from compared groups. Statistical significance was assumed when $p<0.05$ unless stated otherwise.

RESULTS

Islet Isolation

Table 2-1 summarizes the results of islet isolation from all groups. The purity of the isolated islets was close to or exceeded 90% in all groups. The islet yield (IE per gram pancreas) was 1374±191 in group 1, increased to 2236±328 in group 2 (versus group 1, $p=0.06$), and this was further optimized to 2515±291 in group 3 (versus group 1, $p<0.002$). Table 2-2 shows the size distribution of isolated islets. The mean islet size (μm in diameter) was 103.4±1.7 in group 1, 107.6±1.8 in group 2, and 113.3±2.9 in group 3 ($p<0.05$, versus group 1).

Autograft Function

Autograft functions were compared between group 1 (manual dissociation) versus group 2 (automated dissociation). In all autotransplant studies, fasting normoglycemia (PG <150 mg/dL) was restored by 1 week and this was well maintained in a follow-up of 2 months (Figure 2-2). Splenectomy at the conclusion of each study could induce prompt hyperglycemia which confirmed the total dependence of normoglycemia on the viable islet autografts in the apancreatic dogs. Figure 2-3 shows that glucose tolerance was well maintained at IVGTT in all autograft recipients. The K value was 2.0±0.2% (group 1) versus 1.6±0.2 (Group 2) with maximal stimulated insulin releases (μU/mL) of 19.0±2.8 and 15.7±2.2 respectively.

DISCUSSION

Technical improvements of digestion and dissociation in the mass isolation of islets from the pancreases of human organ donors have promoted studies of pancreatic islet transplantation in insulin-dependent diabetes mellitus (14). However, results of renewed clinical trails suggest that yields of highly purified viable islets have to be maximized to reverse insulin-dependent diabetes as well as to overcome the potential reduction of islet graft viability that is caused by deleterious effects, such as heterotopic sites (15), diabetic state (16), immunosuppressive drugs (17), autoimmune injury, and alloimmune rejection. Recently an automated method was introduced and succeeded in mass islet isolation in humans or large animal models (6-10). We improved canine islet isolations by combination of the automated method with our collagenase perfusion technique, however the yield and islet size failed to reach statistical significance when compared with our manual method (7). To improve the isolation techniques we have modified the automated protocol in the present study to maximize the mass isolation of the islets from canine pancreas.

As compared with the manual dissociation method, either of the automated protocols could increase the isolated islet yield by 60-80%. In Agreement with our previous study (7), group 2 automated protocol (with our old shaking technique) tended to enhance the isolation yield and islet size, which compared favourably with the manual method. When a more gentle shaking technique was employed in group 3, the islet size and yield was further increased and compared significantly better than manual method.

The key modifications in our automated protocol compared with Ricordi's are as follows. Firstly the ductal collagenase perfusion was employed in our protocol. The mechanisms of adhesion between islets and exocrine pancreas are critical in pancreas dissociation procedures. The canine and human islets are largely enveloped by a basal membrane and the integrating forces therefore depend upon the cell-to-matrix adhesions

(18). Compared with loading collagenase by static ductal injection (2,6,7), continuous ductal collagenase perfusion may allow a better delivery of collagenase to the peri-insular extracelluar matrix and thereby may improve the enzymatic cleavage of islets from the exocrine tissue. In an earlier study we found that ductal collagenase perfusion was superior to ductal injection for isolating islets from human pancreas (4). Secondly, we used more gentle shaking techniques during the automated dissociation procedure to minimize the mechanical trauma to the islets. The data in this study shows that the gentle shaking technique enhanced the islet yield and mean islet size which compared favourably with non-gentle shaking automated group and significantly better than manual group. Finally, we modified the automated method by using a shaking chamber made of glass instead of steel (6,7). In the procedure of automated isolations, the end point of digestion/dissociation was often under or over estimated due to an unrepresentative sampling. The transparent glass chamber has the advantage in allowing the shaking to be adjusted so that all the tissue in the chamber is stirred up evenly at sampling without overshaking. We also found that it was very important to refer to the fineness of tissue particles in the chamber when determining the end point microscopically. The procedure of tissue dissociation can be visually monitored by using a glass chamber.

In the study of in vivo function, autografts of the islets isolated by manual or automated methods (group 2) could reverse the total pancreatectomy-induced diabetes, and the glucose tolerance was well maintained. Because of a small number of autograft studies in the manual group, statistical tests could not be performed for comparisons of graft function with the automated groups. However manually isolated islets tended to have a higher insulin secretion. Gentle techniques therefore should be emphasized to minimize the shear force during the automated dissociation. Prolonged enzymatic digestion in automated method

(usually 15-20 min longer than manual method) might be an another detrimental factor to islet viability, therefore the digestion time should also be minimized.

In summary, this study describes a modified automated protocol for mass isolation of canine islets, and demonstrates that a gentle automated method combined with ductal collagenase perfusion could improve the recovery of highly purified viable islets from pancreas and increase the islet size as well, when compared with the manual method. The modified automated protocol has been routinely used in our clinical trials and improvements have been achieved in isolating large amounts of highly purified viable islets from human pancreas.

TABLE 2-1

ISLET PURITY AND YIELD BY DIFFERENT PROTOCOLS

		Pancreas Weight	Islet Purity	Mean Islet Yield (±SE)	
Group	n	(g)	(%)	Total (IE)	IE/g
1	15	42.8±1.5	89.3±1.7	57,539±7066	1374±191
2	26	43.8±1.3	90.8±1.3	93,511±11,654	2236±328
3	13	49.5±2.7	89.2±1.2	120,256±10,868	2515±291*

IE = islet equivalent

* = $p<0.002$, compared with group 1

TABLE 2-2

DISTRIBUTION OF ISLET SIZE

		Islet size in μm (% of total islets)					Mean size
Group	n	51-99	100-149	150-199	200-249	>250	Diameter (μm)
1	15	54.7±2.3	35.2±1.7	8.7±1.2	1.3±0.3	0.1±0.1	103.4±1.7
2	26	43.8±2.0	40.6±1.3	8.9±1.2	2.1±0.1	0.1±0.1	107.6±1.8
3	13	45.2±2.6	38.4±1.7	11.6±1.3	4.2±0.8	0.6±0.4	113.3±2.9*

* = $p<0.005$, compared with group 1

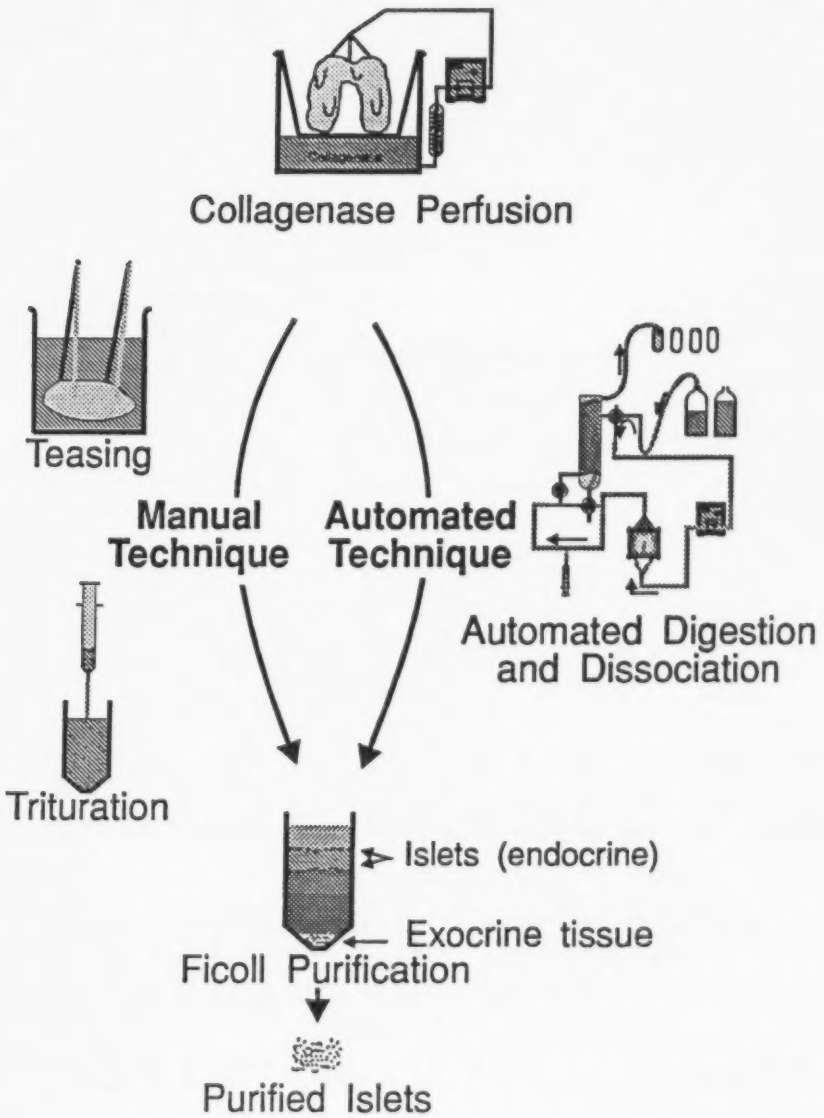

FIGURE 2-1: Schematic of manual and automated isolation protocols

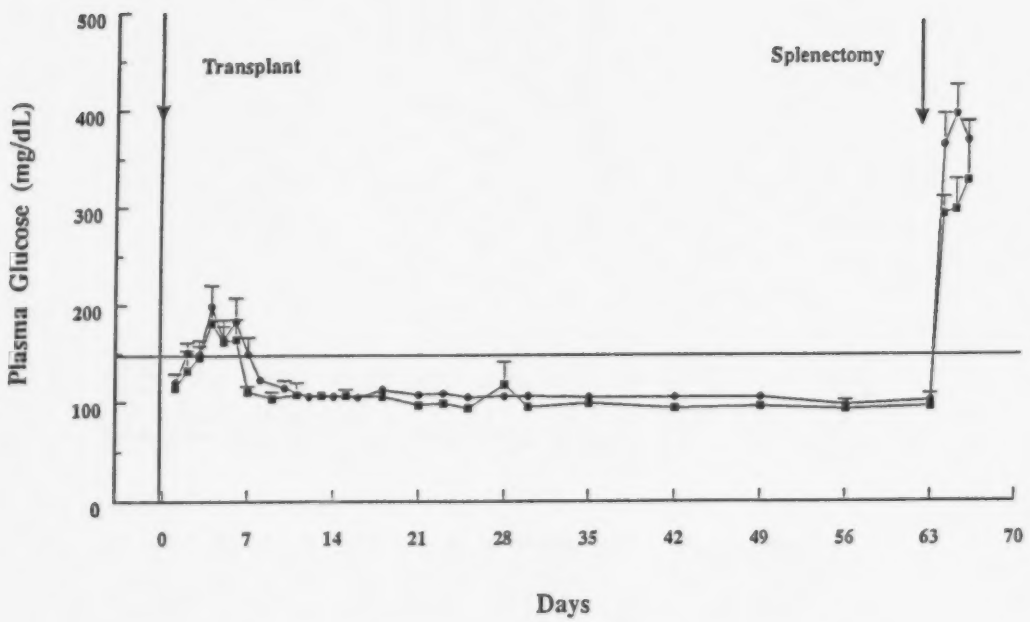

FIGURE 2-2: Fasting plasma glucose after autotransplantation of islets into the spleen of group 1 (n=3, ■------■) and group 2 (n=7, ●------●) dogs. Prompt hyperglycemia was observed after splenectomy.

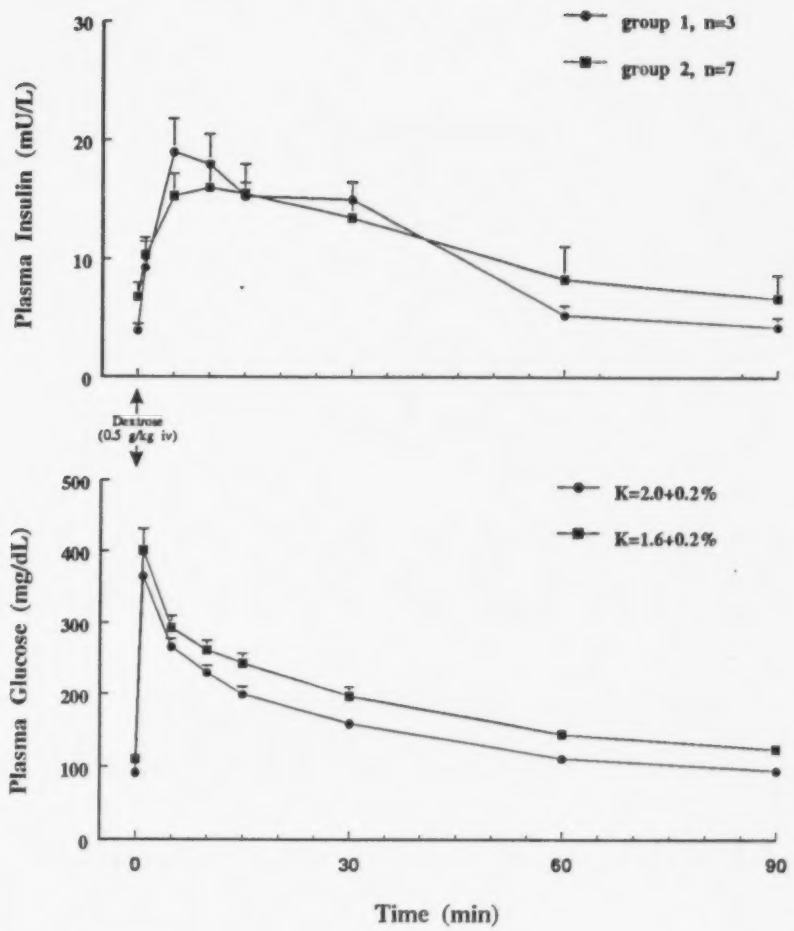

FIGURE 2-3: Plasma insulin and glucose response at intravenous glucose tolerance tests in dogs that received intrasplenic islet autografts.

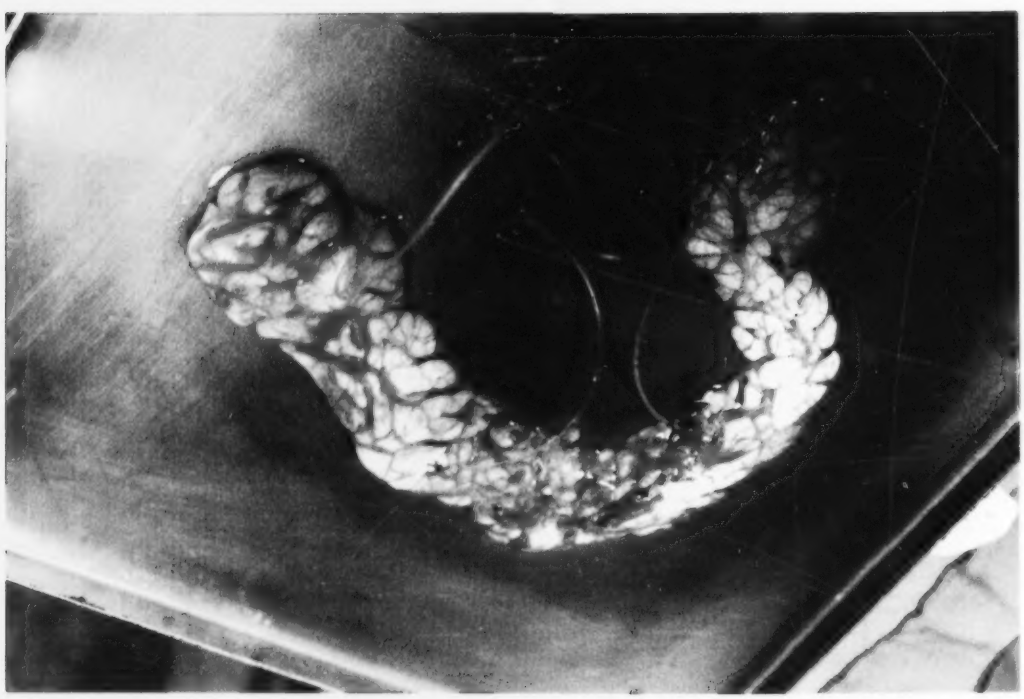

PLATE 2-1: Photograph showing the cannulation of the pancreatic duct. the pancreas was distended by ductal injection of collagenase.

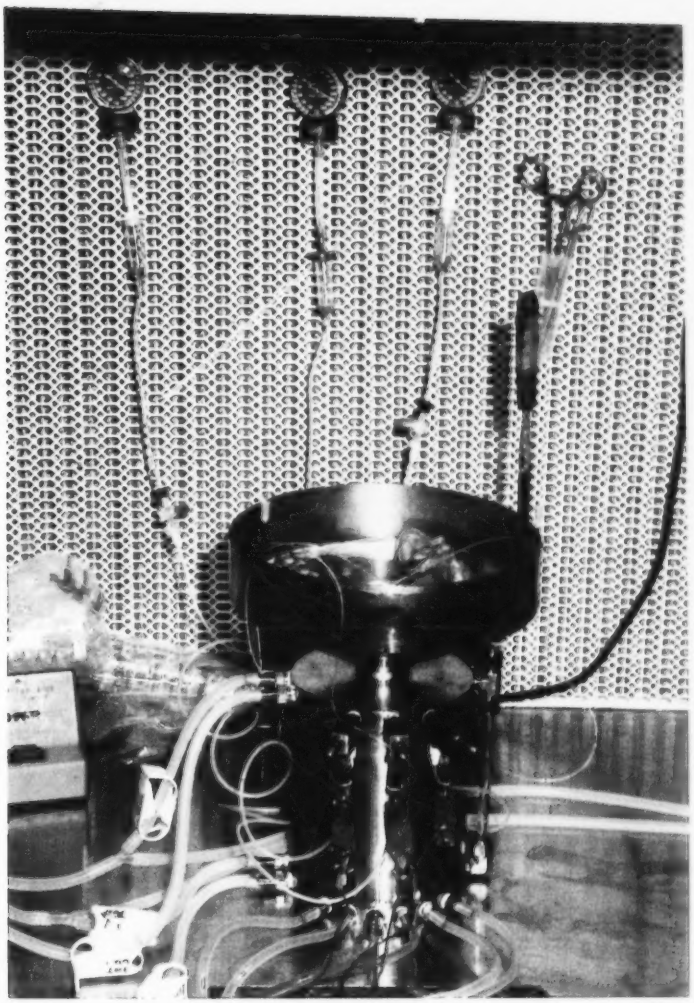

PLATE 2-2: Photograph of a recirculating perfusion apparatus. Temperature and pressure are monitored during the collagenase ductal perfusion of the pancreas.

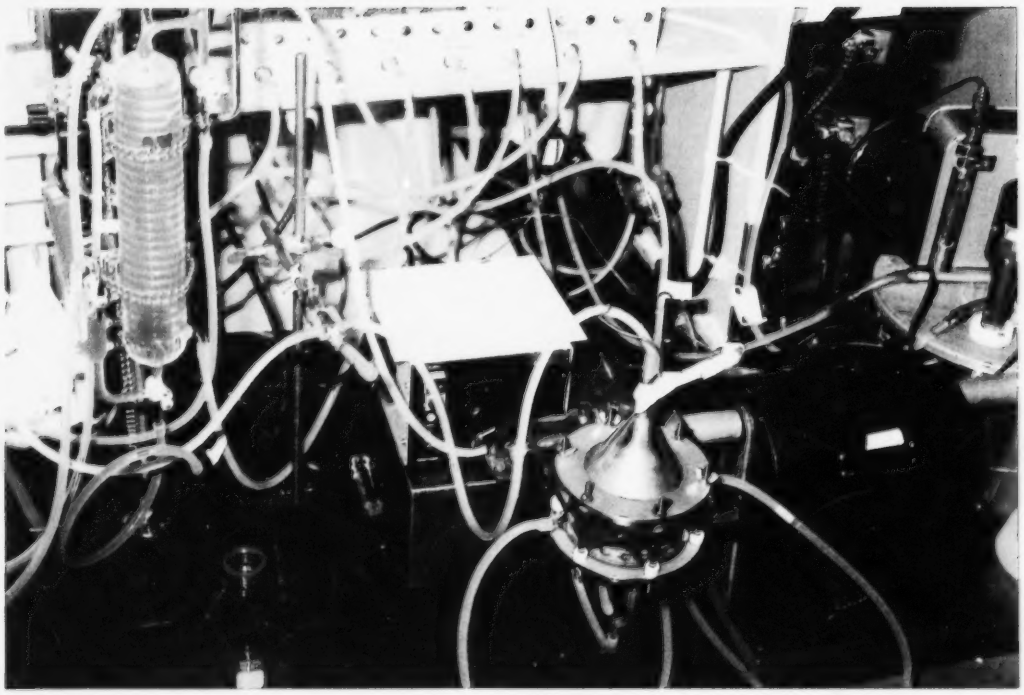

PLATE 2-3: Photograph of an automated tissue digestion/dissociation chamber which connects to a heating/cooling column.

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CHAPTER 3

A COMPARISON OF FICOLL AND DEXTRAN FOR DISCONTINUOUS DENSITY GRADIENT PURIFICATION OF CANINE ISLETS

INTRODUCTION

Many density gradient media have been used for islet purification, including percoll(1), Ficoll (2,3), sucrose (4), dextran (5), metrizamide (6) and bovine serum albumin (BSA) (7). The three more commonly used gradient media for human and animals at present are Ficoll (8-11), Dextran (5,12) and BSA (7).

Ficoll, an expensive copolymer of sucrose and epichlochydrin with a molecular weight of 400,000, has been most widely used for islet purification. However some investigators have reported the findings of its toxicity to the islet cells (3,13). Bovine serum albumin (BSA) was recommended for its success in islet purification (7), but its animal origin may transmit infection, and therefore its clinical application has been limited. Dextran is a polysaccharide with a molecular weight of 72,000 and clinically used as a plasma substitute. Dextran is desirable for its low cost and high efficacy in islet purification in animal models (5,12,14). In comparison with Ficoll, Dextran density gradient provided a higher islet recovery and less toxicity in rodents (14-16). This comparison, however, has not been performed in preclinical models and humans, and the effects of Dextran on the metabolic function of islets have not been well detailed. In this study we compared the discontinuous density gradients of Ficoll versus Dextran for canine islet isolation, as well as their effects on the islet functions in vitro and in vivo.

A version of this chapter has been published in Transplant. Proc. 24:2788,1992

MATERIALS AND METHODS

Isolation of Islets

Twenty pancreases of adult mongrel dogs (body weight 18-25 kg) were mobilized with all major vascular connections maintained until excision of the gland. The right and left branches of pancreatic duct were cannulated in situ using PE90 cannulae, and an extra cannulation was made into the left duct via a cut-down 8 cm from the distal end. The islet isolation procedure was similar to the automated isolation protocol described in Chapter 2. Briefly the pancreas was injected with 50 mL of collagenase (type V, Sigma Chemical Co., St. Louis, MO, 2mg/mL in HBSS) into each cannula and perfused at 4°C for 10 min, and the temperature of perfusate was increased to 30°C. Then the gland was immediately transferred to the automated shaking chamber for continuous digestion and dissociation. The end point of digestion was determined by microscopic examination of the tissue. The digested tissue was washed x3 by cold HBSS, combined and resuspended in chilled Medium 199 in a ratio of 0.6 gram tissue to 1 mL Medium 199.

Purification

The digested tissue was purified with discontinuous density gradient of Ficoll (Fc, n=13) (8) or Dextran (Dx, n=7) (12). Ficoll (MW 400,000, Sigma Chemical Co., St. Louis, MO) was adjusted to pH 7 before autoclave sterilization. Four mL aliquots of tissue suspension were vortexed with 21 mL of Ficoll (density 1.100) contained in 50 mL conical tubes, and 5 mL of Ficoll (density 1.085, 1.075, and 1.045) were overlaid successively. The tissue was then centrifuged at 1,500 rpm for 25 min at 22°C. Dextran (clinical grade, MW 72,000, Sigma Chemical Co. St. Louis, MO) was adjusted to pH 7.4 before autoclave sterilization at 100°C for 15 min. The 4 mL of tissue suspension in each conical tube was spun and the pellet was vortexed with 12 mL Dextran stock solution (density 1.104), overlaid with 5 mL stock solution, 5 mL Dextran of 1.085, and 10 mL Dextran of 1.075 and 1.041

respectively. The Dextran gradient was centrifuged at 400 rpm for 4 min initially and 2,000 rpm for 16 min thereafter. The purified islets in both groups were harvested from the interfaces of upper layers, and washed with Medium 199 x3.

Quantification

Quantification of islet mass and assessment of islet purity were same as described in Chapter 2, which were in accordance with the criteria established at the 1989 International Workshop On Islet Assessment (17).

Autotransplantation

Purified islets from Fc group (n=7, 3,679+623 IE/kg body weight) and Dx group (n=6, 5,527+233 IE/kg) were autotransplanted into the spleen by venous reflux. The fasting plasma glucose (PG) was measured three times a week in a follow-up of 2 months with a glucose oxidase method (Type II Glucose Analyzer, Beckman Co, Fullerton, CA). Intravenous glucose tolerance tests (IVGTT) were performed at 8 weeks posttransplant. The K values (decline of plasma glucose, %/min) (18) and plasma immunoreactive insulin (IRI) were measured as described in Chapter 2 (19). At the conclusion of the follow-up, general anaesthesia and laparotomy were performed on autograft recipients in both Fc and Dx groups. Blood was sampled simultaneously from the splenic vein and artery before and after intravenous glucagon challenge (1mg/20kg) for assay of IRI to determine the veno-arterial insulin gradient. Following the sampling procedure the spleen bearing islet grafts was removed and the animals were returned to the vivarium where daily PG was monitored for at least 3 days.

Perifusion

To compare the in vitro viability of islets purified with Ficoll (n=9) versus Dextran (n=9), paired studies of glucose perifusion were performed following an overnight islet

culture at 37°C. Duplicate aliquots containing 200-300 IE of islets purified with Ficoll or Dextran from the same pancreas (n=9) were perifused at 37°C with glucose concentrations of 50, 500, and 50 mg/dL for 60 minutes at each concentration. The perifusate was collected and the IRI was measured.

Data Analysis

All results were expressed as mean±SE. The two-tailed Students' t-test for paired and unpaired data were used to determine the significance of differences between parameters from compared groups. Statistical significance was assumed when $p<0.05$ unless stated otherwise.

RESULTS

Islet Isolation

Table 3-1 summarizes the results of islet isolation profile. The isolation yield (number of islets equivalent (IE) to 150 μm in diameter per gram pancreas) was 2,515±291 in Fc group versus 2,441±584 in Dx group ($p>0.5$). The purity (%) of the isolated islets was 89.2±1.2 in Fc group and 90.7±1.5 in Dx group respectively ($p>0.5$, Fc vs Dx). The mean islet size (um in diameter) was 113.3±2.9 in Fc group versus 115.2±5.3 in Dx group ($p>0.5$). Compared with prepurification islet count, the recovery of islets isolated with Ficoll and Dextran was 55.6% and 57.3% respectively.

Autograft Function

Autograft function of islets isolated with Ficoll and with Dextran were compared by measurement of postoperative daily fasting PG and IVGTT. Fasting normoglycemia (PG<150mg/dL) in both groups was restored by one week, and this was well maintained during a follow-up of 2 months (Figure 3-1). Splenectomy at the conclusion of each study

induced prompt hyperglycemia which confirmed the total dependence of normoglycemia on the viable islet autografts in the pancreatectomized recipients. Figure 3-2 shows that glucose tolerance was well maintained at IVGTT in both groups. The K value was 1.6±0.2% (Fc) versus 1.7±0.2% (Dx) ($p > 0.5$) with a maximal intravenous glucose-stimulated insulin release (μU/mL) of 15.7±2.2 (Fc) versus 14.1±2.9 (Dx) ($p > 0.5$). After intravenous glucagon challenge the insulin gradient between splenic vein and femoral artery peaked at 100.5±40.9 μU/mL (Fc) and 77.5±21.2 μU/mL (Dx) from a basal veno-arterial insulin gradient level of 47.0±15.2 and 43.3±11.2 respectively ($p > 0.05$ for both stimulated and basal veno-arterial gradients). (Figure 3-3)

Function In Vitro

Figure 3-4 shows the results of perifusion of the islets purified with Ficoll or Dextran from the same pancreas. The basal insulin secretion (μU/islet/min) was 0.023±0.007 (Ficoll) and 0.043±0.018 (Dextran) and increased significantly after exposure to high glucose (500 mg/dL) to 0.068±0.019 and 0.115±0.039 respectively. No significant differences were found between the two groups. The stimulated insulin release in both groups was biphasic and the basal secretion was restored when glucose concentration was returned to 50 mg/dL.

DISCUSSION

The possible advantages of transplanting highly purified islet preparation include increased safety, improved islet engraftment and reduced immunogenicity of the grafts. Unpurified islet grafts may cause severe postoperative complications such as portal hypertension, disseminated intravascular coagulation, and sepsis (20-22). Exocrine contamination impaired engraftment of isogeneic rat islets in renal subcapsule (23) and in liver (24). Contaminating exocrine and lymphatic tissue enhance the immunogenicity (25) and accelerate the rejection of islet allografts (26).

The purification of larger numbers of viable islets has proved difficult, and this is particularly true for human islets. Proper choice of gradient purification medium is one of the important factors in successful islet isolation. There are many gradient media for islet purification, among which Ficoll and Dextran have been widely used for their high purification efficacy, non-animal origin, and ability to be autoclaved. Some studies found that Dextran discontinuous gradient, compared with Ficoll, provided higher islet isolation recovery and less cytotoxity in rodents (16-17). However this comparison has not been studied in preclinical animal models and humans, and the effects of Dextran on the metabolic functions of isolated islets have not been well detailed.

The data in this study shows that in comparison with Ficoll gradient purification, Dextran demonstrated an equivalent efficacy for islet purification with similar isolation yield, purity, and recovery. Autotransplant study showed that Dextran isolated islet grafts had a similar performance to Ficoll in maintainance of normoglycemia and IVGTT. In the paired perifusion study in both groups, about three-fold of glucose-stimulated insulin release was obtained with a biphasic response after exposure of islets to high concentration glucose, and the basal insulin release level was restored after glucose returned to low concentration. This indicates that either Ficoll or Dextran isolated islets have good in vitro viability. In the perifusion study we found that Dextran isolated islets tended to have a higher insulin release compared with Ficoll, but the difference failed to reach statistical significance ($p > 0.05$). On the other hand, the data in IVGTT study showed that the Ficoll isolated islet autografts tended to have a higher insulin secretion than thoses isolated with Dextran. Considering its reduced cost (one-third as Ficoll) and equivalent purification efficacy compared with Ficoll, Dextran may be a more suitable medium for large scale islet isolation in large animals and humans.

TABLE 3-1

PROFILE OF ISLET ISOLATION WITH FICOLL VERSUS DEXTRAN

	Ficoll (n=13)	Dextran (n=7)
Islet Purity (%)	89.2±1.2	90.7±1.5
*Pre-Yield (°IE/g)	4,523±733	4,207±981
**Post-Yield (IE/g)	2,515±291	2,411±584
Islet Recovery (%)	55.6	57.3
Mean Islet Size (μm)	113.3±2.9	115.2±5.3

°IE/g : number of islets equivalent to 150μm in diameter per gram pancreas.
*Pre- : pre-purification.
**Post-: post-purification.

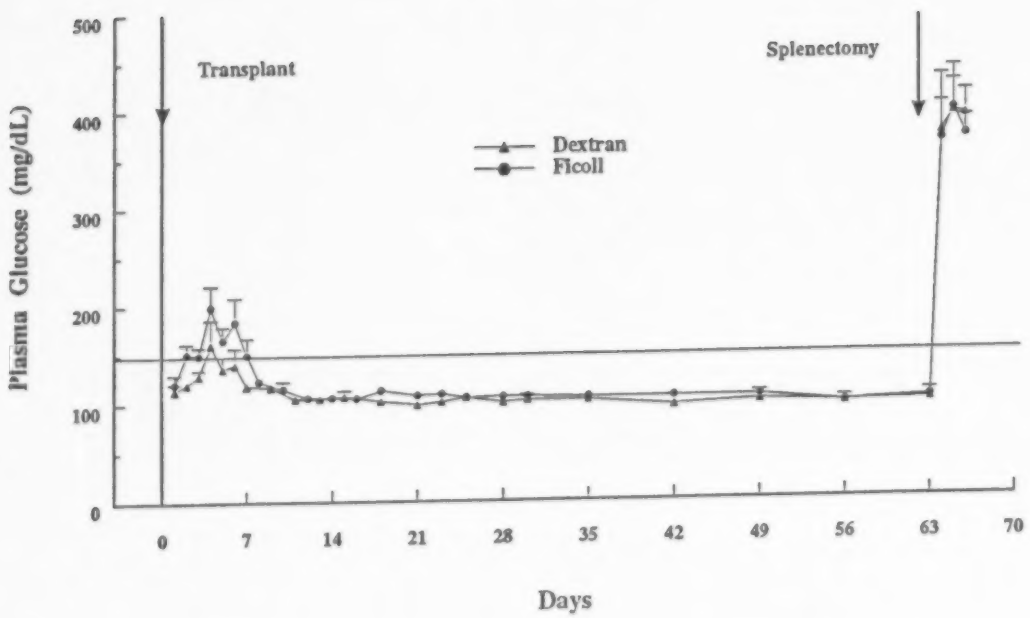

FIGURE 3-1: Fasting plasma glucose after autotransplantation of islets into the spleen of Ficoll group (n=7) and Dextran group (n=6) dogs. Prompt hyperglycemia was observed after splenectomy.

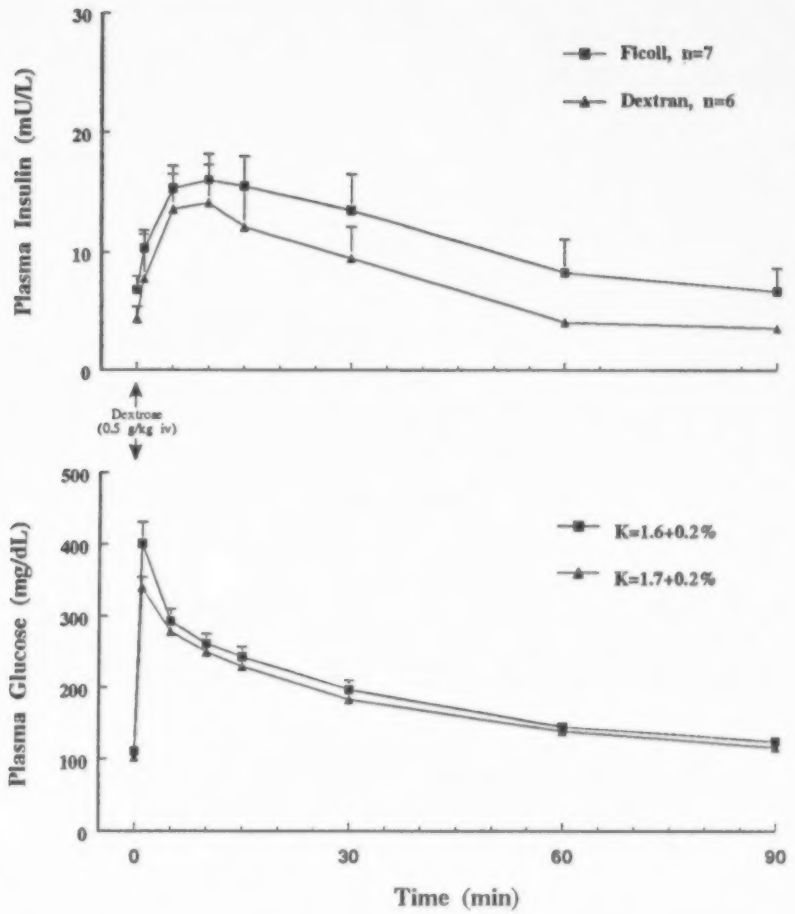

FIGURE 3-2: Plasma insulin and glucose responses at intravenous glucose tolerance tests in dogs that received intrasplenic islet autografts.

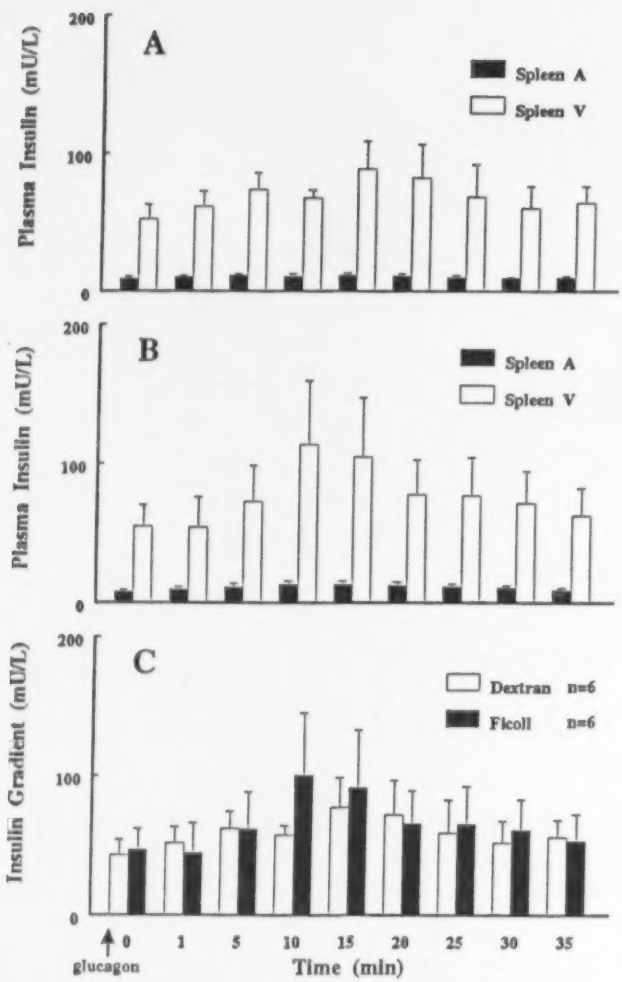

FIGURE 3-3: Insulin levels in the splenic vein and femoral artery of the dogs that received Dextran purified islets (graph A) or Ficoll purified islets (graph B). Graph C compares the veno-arterial insulin gradients of Dextran group versus Ficoll group.

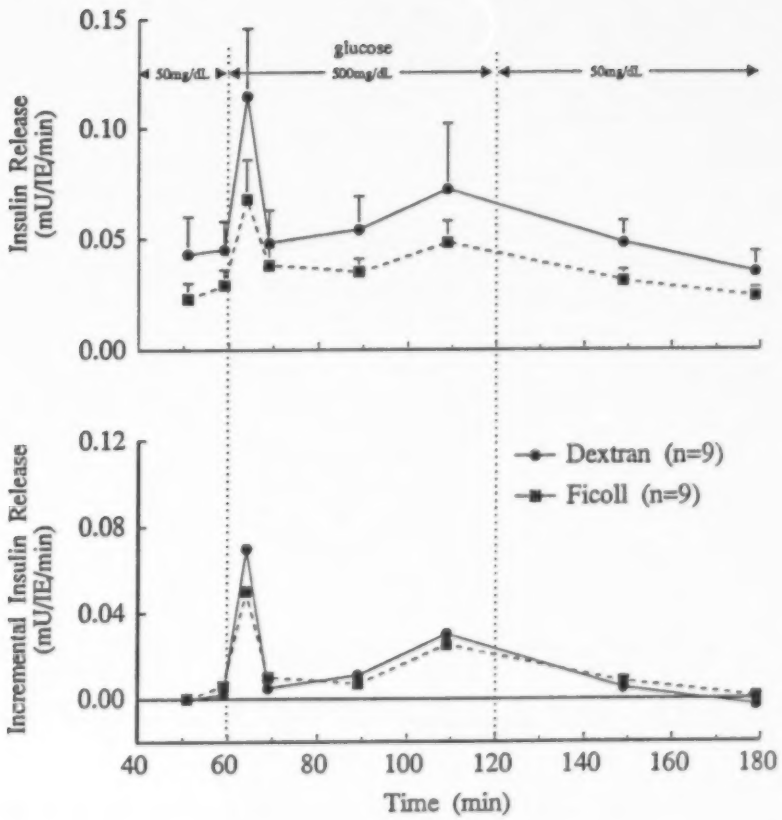

FIGURE 3-4: Insulin secretion by islets purified with Ficoll or Dextran at glucose perifusion.

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CHAPTER 4

SURVIVAL AND FUNCTION OF PURIFIED ISLETS IN THE OMENTAL POUCH SITE OF OUTBRED DOGS[1]

INTRODUCTION

Recent advances in the mass isolation of highly purified islets and in the prevention of rejection by novel immunosuppressive drugs have facilitated experimental islet transplantation in large animals (1,2) and in human subjects (3-6). Many different sites have been used for experimental islet transplantation in rodents, including the liver, spleen, renal subcapsule, peritoneal cavity, muscle, and testis. However, the most success in large animals has been limited to the spleen by injection into the pulp (7) or venous reflux (8), and more recently via the portal vein to the liver (9). However, islet function in the intraportal site is subject to delayed failure during prolonged follow-up (9). The spleen provides more effective long-term function than the liver for equivalent quantities of pure islets (10,11), but failures also occur in this site, and access to the splenic venous site is a more difficult procedure with greater potential for morbidity.

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An ideal site for islet transplantation in humans would be one which affords minimal hazards to the patient, hormonal secretion directly into the portal venous system, and retrieval of the graft for biopsy purposes. An omental site not only fulfils these criteria, but may also facilitate implantation of micro- or macroencapsulated islets. Use of the omental site has been reported for isolated islets in rodents (12) and for fetal pig pancreas in pigs (13). Altman reported infusion of islets into an epiploic artery in human subjects (14). Intraperitoneal injection has been chosen for free islets (15) or microencapsulated islets in rodents (16), and for encapsulated islets in spontaneously diabetic dogs (17), but no attempt was made to localize islets to an omental site. The efficacy of an omental site has not been evaluated in a preclinical large animal model. The aims of this study were to devise an omental pouch implantation site for auto- or alloimplantation of canine islets, to define the threshold of the islet mass required to reverse diabetes in this site, and to compare the function of islets implanted into the omental pouch and spleen.

MATERIALS AND METHODS

Animals and Care

Forty-eight adult conditioned mongrel dogs of both sexes weighing 16-28 kg (donors) or 10-15 kg (allograft recipients) were used for the study. A veterinarian supervised their overall care. Dogs were housed in a large animal vivarium and attended in accordance with the recommendations of the Canadian Council on Animal Care. Surgical procedures were performed under general aesthesia with Pentobarbital. The dogs were weighed twice weekly and permitted unrestricted exercise. Full daily diet consisted of 360 g of meat (Dr. Ballard's) and 600 g of Burger Bits supplemented postoperatively with eight capsules of Cotazym (Organon Canada Ltd., West Hill, ON).

Isolation and Quantification of Islets

The pancreas was mobilized with all major vascular connections maintained until just before excision of the gland. The gland was excised, weighed, and ducts cannulated. Highly purified islets were isolated by perfusion of collagenase (2 mg/mL, Type V; Sigma Chemical Co., St. Louis, MO) via the duct, automated dissociation, and discontinuous density gradient purification with Ficoll, as previously described (18). The islet mass was quantified and purity assessed in accordance with criteria established at the 1989 International Workshop on Islet Assessment (19). Briefly, aliquots from the final islet suspension were incubated with Dithizone, and examined microscopically with the aid of an optical graticule. The crude number of islets in each diameter range (60-99, 100-149, 150-199, 200-249, >250 μm) were counted by two individual investigators and the total in each range was converted to the number of islets equivalent (IE) to 150 μm in diameter. The number of IE was then multiplied by the volume of a 150 μm sphere (0.001767 μL) to calculate the graft volume. Purity for insulin-containing islets was estimated by comparing the relative quantities of Dithizone stained and unstained tissue at microscopy.

Transplantation

Defined quantities of freshly-isolated islets were grafted into totally pancreatectomized outbred mongrel recipients. For recipients of intrasplenic islet autografts (n=12), the islets were refluxed into the splenic vein as previously described (8). For omental pouch recipients (n=12 autografts and n=12 allografts) the greater omentum was mobilized and folded upon itself, creating a circular pouch of ~10 cm diameter. The margins of the omentum at the periphery of the pouch were sutured together with 3-0 polyglycolic acid sutures, care being taken to preserve the gastroepiploic arterial arcade. A 40 cm length of PE90 tubing was attached to a 20 gauge needle to form a catheter, then advanced into the

pouch. Islets were suspended in 5.0 mL of tissue culture Medium 199 and slowly injected into the pouch via the catheter, and the catheter was then removed.

For each allograft, islets from single donors were implanted. A total of three dogs received no immunosuppression and nine dogs received Cyclosporine A (CsA) at a dosage of 5-15 mg/kg administered intramuscularly daily for 4 days before and 30 days after transplantation. Whole blood CsA trough levels were measured daily by monoclonal RIA (Cyclo-trac SP RIA kit, Incstar Corp., Stillwater, MN).

Evaluation of graft function: Fasting plasma glucose (PG) was monitored daily using a glucose oxidase assay (Beckman Type 2 Glucose Analyzer, Fullerton, CA). The graft was deemed to have failed when PG rose from initial normoglycemia (<150 mg/dL) to >200 mg/dL for 3 consecutive days. Intravenous glucose tolerance tests (IVGTT, 0.5 g Dextrose/kg body weight) were performed at 1 month and 2 months postoperatively. K values (decline of glucose, %/min) during IVGTT were calculated from PG levels at 5, 10, 15, and 30 minutes after injection of Dextrose (20). Plasma was collected simultaneously for assay of immunoreactive insulin (IRI) using double-antibody RIA with human insulin standards (Pharmacia, Uppsala, Sweden) (21). At the conclusion of follow-up of omental autograft recipients, general aesthesia was administered and the portal vein and femoral artery were cannulated. Blood was sampled simultaneously from the vein and artery before and after intravenous glucagon (1 mg/20 kg) for assay of PG and IRI. Following the sampling procedure, the greater omentum bearing the islet graft was detached from the stomach and excised, and the animals were returned to the vivarium where PG was monitored daily.

Histologic studies: The omentum bearing transplanted islets was fixed in formaldehyde. Representative samples were removed and embedded in paraffin blocks for sectioning and immunoperoxidase staining for insulin with avidin-biotin-peroxidase complex

method (ABC Standard Kits, Vector Lab Inc, Barlingame, CA). Normal dog pancreas was used as control.

Data analysis: All results were expressed as mean±SE. The two-tailed unpaired Student's t-test was used to determine the significance of differences between parameters from different groups. Statistical significance was assumed when $p<0.05$ unless stated otherwise.

RESULTS

Graft preparation: Isolation of islets in these studies resulted in a yield of 2125±159 purified IE/g of pancreas. The mean total islet recovery was 101,188±7489 IE/pancreas and purity for insulin-containing islet tissue was 89.6±1.1%. The final packed cell volume implanted was <0.5 mL. The mean islet diameter was 110.8±1.7 μm and islet size was distributed as 45.7% <100 μm, 50.8% at 100-200 μm, and 3.5% at >200 μm.

Graft recipients: Normoglycemic dogs maintained their body weight after transplant: in the splenic autograft group, the body weight was 19.1±0.6 kg before and 20.0±0.7 kg after transplant, and for the omental group, body weight was 20.3±0.5 before versus 21.4±0.3 kg after transplant. In the allograft group, the body weight was also maintained: 12.0±1.2 versus 11.9±0.9 kg. In dogs that developed fasting hyperglycemia, weight was stabilized by administration of exogenous insulin for an additional 2 weeks until euthanasia. No complications were attributable to the transplant procedures. In one dog that received an allograft, some of the islets, estimated to be 50% of the graft, spilled out of the pouch into the general peritoneal cavity during implantation.

Dose-response studies: Table 4-1 summarizes the success rates (daily fasting glucose <120 mg/dL for 2 months) were investigated after incremental volumes of purified islets were autoimplanted into the spleen or omental pouch. In the spleen group, the threshold

of islet mass that consistently reversed pancreatectomy-induced diabetes was 4 μL (2264 IE)/kg body weight: one of four dogs that received <4 μL/kg and all eight that received >4 μL/kg (range 4-11.9, mean 6.3±1.1) were normoglycemic for 2 months. In the omentum group, only one of seven dogs that received <10 μL (5650 IE)/kg (range 4-9.8, mean 7.5±0.9) was normoglycemic for 2 months; the others that received this islet mass were initially normoglycemic, but this persisted for only 18±7 days and all had become diabetic by 33 days. In contrast, when >10 μL/kg (range 10-12.5, mean 11.3±0.5) was implanted into the omentum, all 5 dogs maintained normoglycemia during follow-up for 2-3 months.

Metabolic studies: Figure 4-1 demonstrates a comparison of the daily fasting glucose levels in dogs that had successful intrasplenic (6.0±0.9 μL/kg, n=9) or intraomental (10.8±1.5 μL/kg, n=6) islet grafts. During the fourth, fifth, and sixth days, transient hyperglycemia was observed in both groups, this being more marked in the omental group. All dogs became normoglycemic (PG <150 mg/dL) by 1 week with similar fasting PG values in both groups. In the spleen group, this was maintained until splenectomy was performed at 2 months. In the omental group, one graft failed at 63 days, resulting in hyperglycemia. Laparotomy revealed that the omental sutures had disrupted so that the pouch harbouring the islets had come apart. The remaining 5 dogs remained normoglycemic at 3 months follow-up, at which time laparotomy revealed an intact pouch. The plasma insulin levels in the portal vein and femoral artery of all six omental pouch dogs were assayed: in two instances, technical problems with placement of the cannula precluded interpretation of the data, but results in the other four, including the dog with the disrupted pouch, are shown in figure 4-2. Insulin levels in the portal vein exceeded those observed in the systemic circulation and tended to rise further after administration of glucagon, although the increase did not reach statistical significance. Omentectomy at 3 months in all five long-term normoglycemic dogs resulted in prompt hyperglycemia.

The preoperative levels of insulin during IVGTT showed basal IRI (mU/L) of 4.8±0.4 and peak IRI of 41.2±3.1 with a K value of 2.4±0.1% (n=5). At 2 months follow-up, the insulin and K values had declined in both omental and splenic groups to the values shown in figure 4-3. The basal IRI level (mU/L) before Dextrose injection was 2.0±0.4 in the omentum versus 6.1±1.0 in the spleen ($p<0.01$) and IRI peaked to 5.5±0.7 in omentum versus 13.7±2.1 in spleen ($p<0.01$) at 10 minutes after intravenous Dextrose challenge. PG levels declined at similar rates in both groups, resulting in K values of 1.3±0.4% in omentum versus 1.5±0.2% in spleen ($p>0.2$). PG did not return to basal levels in the omentum group, and 60 and 90 minute levels were significantly higher than the spleen group at these times.

Histology: Microscopic evaluation of the omental graft sites showed evidence for revascularized islets with granulated Beta cells. Islets could be identified by immunoperoxidase insulin staining between mesothelial-lined surfaces of the omentum. Other islets were found embedded within omental fat (Plate 4-1).

Allograft function: An islet mass that exceeded 10 μL/kg was implanted in all instances to ensure initial normoglycemia. All recipients became initially normoglycemic. Nonimmunosuppressed controls had a mean duration of normoglycemia of 4.3±1.8 days, then became persistently diabetic for 2 weeks, requiring daily insulin therapy to control fasting hyperglycemia. All nine dogs that received CsA were normoglycemic by 7 days. In the single dog in which the islets had spilled out of the pouch into the peritoneal cavity at implantation, fasting glucose rose to 200-250 mg/dL at 8 days and remained in this range for 3 weeks. In the remaining eight dogs, the duration of normoglycemia was prolonged. In three of these dogs, lower initial trough whole blood levels of CsA (500-750 ng/mL) were observed and their grafts failed at 10, 15, and 21 days. Figure 4-4 shows the results in the other five dogs that had CsA levels of 1000-1200 ng/mL during the first 10 days and ~600 ng/mL thereafter. In these dogs, normoglycemia was maintained for the duration of CsA therapy. Normoglycemia

persisted for a total of 34±9.5 days after cessation of CsA. Metabolic studies in these dogs showed that K values at 30 days had declined to 0.9±0.3% from levels of 2.4±0.1% preoperatively. Basal PG levels in the allograft recipients were similar to those in the autograft group. K values in three dogs that remained normoglycemic for 4 weeks after cessation of CsA improved from 0.7±0.2% before stopping CsA to 1.0±0.3%.

DISCUSSION

These data demonstrate prolonged function of purified islets in the omental pouch when a sufficient mass of islets was provided. When a mass of islets exceeding 10 μL/kg was autoimplanted (n=5) or alloimplanted (n=12) into the pouch, normoglycemia was induced in all. In contrast, only one of seven that received an islet mass <10 μL/kg became normoglycemic. The overall success rate (normoglycemia) of transplantation of islets into this site in the present study was therefore 17 of 24 dogs or 71%, which compares favourably with alternative sites reported in dogs (2,7-10). Some technical factors appeared to be critical to ensure prolonged function of islets in the pouch. First, loss of integrity of the pouch appeared to be a possible factor contributing to early graft failure in one autograft recipient that developed hyperglycemia at 63 days. In this dog, the pouch sutures were disrupted, resulting in opening of the pouch. The disruption of the omental pouch may have damaged the capillaries which revascularized the islets, thereby causing islet necrosis. Therefore, nonabsorbable sutures are suggested to maintain integrity of the pouch more effectively. In another instance, an allogeneic graft that contained sufficient islets became dysfunctional after the islets spilled out of the pouch into the peritoneal cavity during implantation. While immune injury could not be excluded in this instance, the persistence of some graft function was evident as mild hyperglycemia (PG 200-250 mg/dL) persisted, a finding in sharp contrast to nonimmunosuppressed controls (n=3) and to the early allograft

failures in which PG consistently exceeded 300 mg/dL. This suggests that it was crucial to localize the islets to the omental site. This observation is consistent with earlier observations in rodents that the injection of islets into the general peritoneal cavity is less effective (22) and that higher islet mass of at least 8 μL/300 g (~25 μL/kg) was needed to consistently induce euglycemia in streptozotocin-diabetic recipients (15).

The use of the omental site in this study differs from other reports in rodents and human subjects. In earlier studies in rats, a strip of parietal peritoneum was used to localize the islets to the pouch site (12). In recent investigations with humans, an epiploic artery was used (14). Recent investigation in our laboratory with an epiploic arterial infusion of highly purified islets in dogs led to infarction of the islet grafts and nonfunction (unpublished observations).

In the present study, the critical mass of islets needed in the omental pouch has been compared with the splenic venous site, our standard model (23). The omental site was found to consistently require a greater volume of islets to induce normoglycemia in apancreatic recipients. The threshold of islet mass required in the omentum group exceeded by 2.5-fold that required in the spleen. The reason for this is unclear, but may be attributable to reduced survival of islets in the omental site. Compared with islets in splenic venous sinusoids, omental islets could be expected to have delayed access to nutrients supplied by blood perfusion, thereby reducing islet survival from ischemia. Furthermore, the degree of hyperglycemia during initial engraftment was more marked in the omental group. This was found in earlier studies to reduce blood perfusion of the islets, resulting in impaired engraftment and function (24). Clearly, the use of an increased islet mass was able to overcome this problem, but at the expense of increased islet requirements.

Metabolic studies showed that islet graft function was originating from the omental site. Omentectomy resulted in prompt hyperglycemia and an arteriovenous insulin gradient

was observed in portal vein versus systemic artery. However, function differed significantly from the splenic site. The insulin levels in peripheral blood of omental dogs were significantly lower than those in dogs with intrasplenic islets. Although K values during IVGTT were not significantly different between the two groups, the omentum grafts failed to restore basal glycemic levels by 90 minutes and the 60 and 90 minute PG values in this group were significantly higher than those in the spleen. This may have been attributable to the blunted early insulin release as a result of denervation, reduced islet engraftment, or suboptimal islet mass in the omental site.

The data in the allotransplant study shows that prolonged survival of allogeneic islets can be achieved in this site in CsA-treated recipients. The depth of immunosuppression appeared to be crucial to this success, since prolonged function was observed in dogs that had low initial CsA blood levels, but persistent function was observed when initial CsA levels during the first 10 days exceeded 1000 ng/mL. These requirements for CsA monotherapy are consistent with those reported in earlier studies to induce and sustain function of purified multi-donor islets in the liver (1) or single donor islets to the spleen (25) of pancreatectomized dogs. The present data on the survival of allogeneic islets confirm and expand on the data of Lacy et al. who reported histological but not functional survival of cultured allogeneic islets into an intraperitoneal (mesenteric) site of antilymphocyte-treated dogs (26). In the latter report, the islet mass used was not defined, which may have influenced the survival of lymphocyte-depleted grafts if small quantities of islets were implanted. Further studies using immunomodulated islets in the quantities reported in the present study would be needed to determine the efficacy of donor islet culture.

The survival of intra-omental allografts after cessation of CsA (34±9 days) compares favourably with survival of intrasplenic grafts previously reported from our laboratory (25). In the latter studies, despite higher initial dosages of CsA, the mean survival time of grafts

after cessation of CsA was only 11±2 days. Several factors may account for the more prolonged function observed here. First, higher activities of cell-mediated immune effector responses in the spleen may accelerate rejection of islets in the spleen. Second, it cannot be excluded that a lower clearance rate of the lipophilic CsA from the fatty omentum tissue induces a higher CsA tissue concentration, thereby enhancing survival. This has been an explanation offered for the prolonged survival of cardiac allografts in combination with CsA. Finally, presentation of donor antigen via lymphatics in the omental route may differ from direct intravascular injection in the spleen which may reduce sensitization of the recipients to islet alloantigens. The survival advantage for purified single donor islets observed in the present study remains less than that achieved by Alejandro et al., but in that study Beagle recipients were used, multiple donors were required, and liver site was chosen.

The K values during CsA therapy were identical to those observed in earlier studies of intrasplenic islet allografts reported from our laboratory (25). Although the present study does not specifically address the effects of CsA on islet function, the findings do indicate an adverse effect of CsA on engraftment and/or function of purified islets in the omental site. First, the K values during IVGTT in the omental allograft group were reduced compared with autograft recipients. Second, in the dogs that received CsA, the K values improved when CsA was discontinued. Third, mean PG levels during CsA therapy were 128±1 mg/dL versus 109.0±3.0 mg/dL ($p<0.05$) in autograft recipients who did not receive CsA. Finally, the PG levels were found to decrease significantly to 113±1 mg/dL ($p<0.02$) when CsA therapy was discontinued. These findings of an adverse effect of CsA are in agreement with those reported earlier for purified islets implanted into the liver (27).

The omental site offers several practical and theoretical advantages. The creation of the pouch is technically straightforward and may be amenable to minimally invasive laparoscopic surgery. Insulin and other hormones from the islets are secreted into the portal

venous system. The omentum could be mobilized on its vascular pedicle to an extra abdominal subcutaneous site to facilitate recovery of the islets during biopsy. The pouch may be suitable to localize mass quantities of microencapsulated islets in large animals or humans. Drugs such as capillary growth factors (28) or immunosuppressive agents (29) could be applied directly to the pouch to enhance islet engraftment or minimize systemic effects of antirejection therapy. The present study may serve as a foundation from which to develop the omental pouch site for implantation of mass quantities of purified islets.

TABLE 4-1

NORMOGLYCEMIA AFTER ISLET AUTOTRANSPLANTATION INTO APANCREATIC RECIPIENTS

	GRAFT VOLUME (μL/kg)					
GRAFT SITE	<4	4-5.9	6-7.9	8-9.9	10-11.9	>12
SPLENIC						
Success Rate*	1/4	5/5	1/1	1/1	1/1	
OMENTAL						
Success Rate*		0/2	0/2	1/3	3/3	2/2

* Fasting PG maintained <120 mg/dL at 2 months follow-up

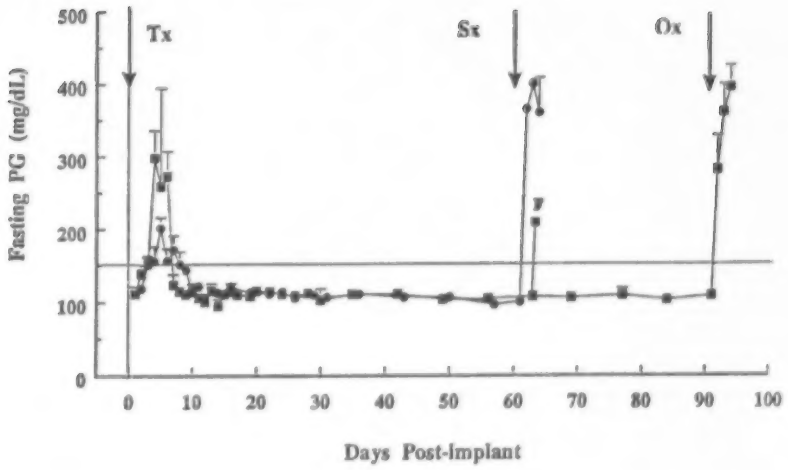

FIGURE 4-1: Comparison of fasting plasma glucose during prolonged follow-up after transplantation (Tx) of islets into the spleen (n=9, ●—●) or omental pouch (n=6, ■—■) of apancreatic dogs. One recipient of omental pouch islets developed graft failure (F) when the pouch became disrupted at 63 days. Prompt hyperglycemia was observed after splenectomy (Sx) or omentectomy (Ox).

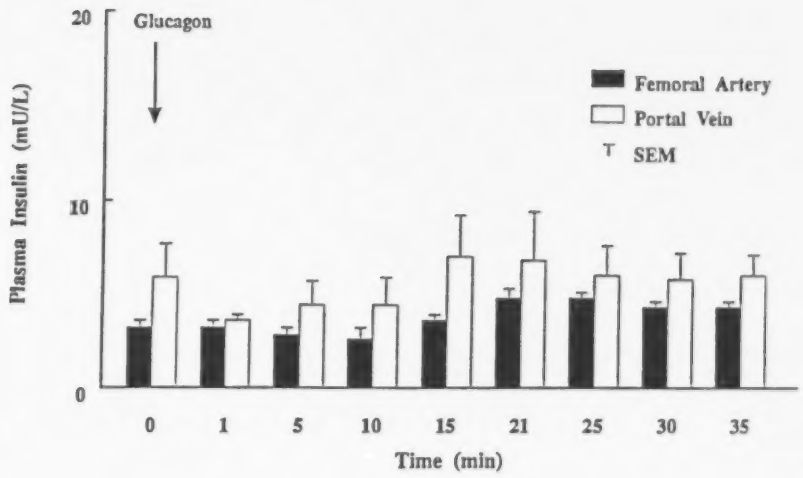

FIGURE 4-2: Comparison of mean (±SE) insulin levels in the portal vein and femoral artery in four dogs bearing islet autografts in the omental pouch at 2-3 months posttransplant.

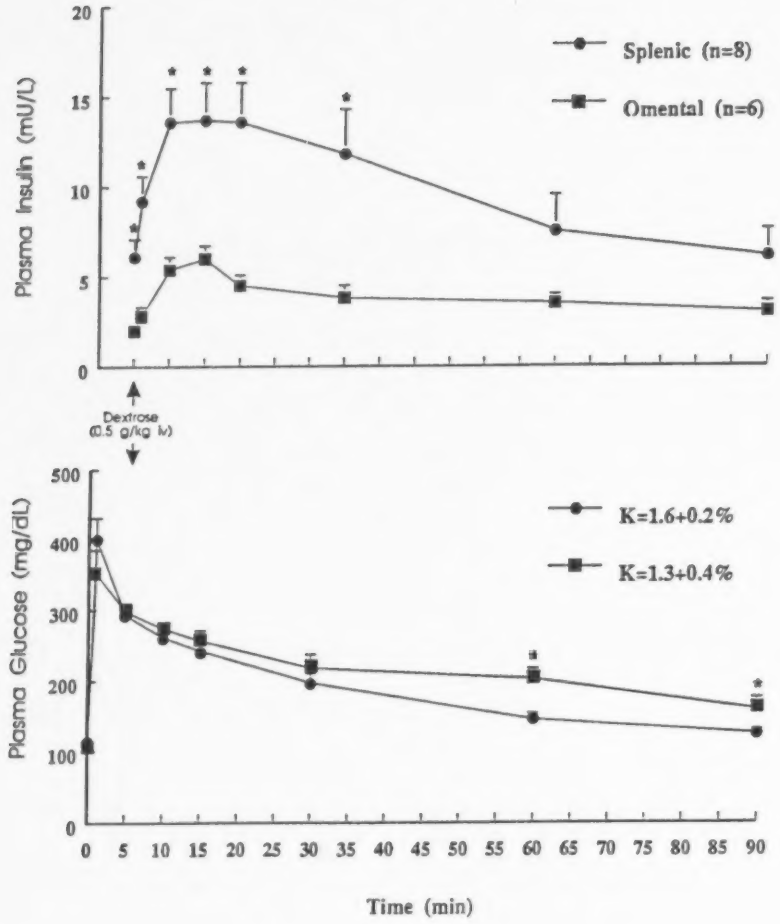

FIGURE 4-3: Plasma insulin and glucose responses to intravenous Dextrose in dogs that received intrasplenic (n=8, ●—●) or omental (n=6, ■—■) islet autografts. The values of "*" denote significant differences between omentum and spleen groups.

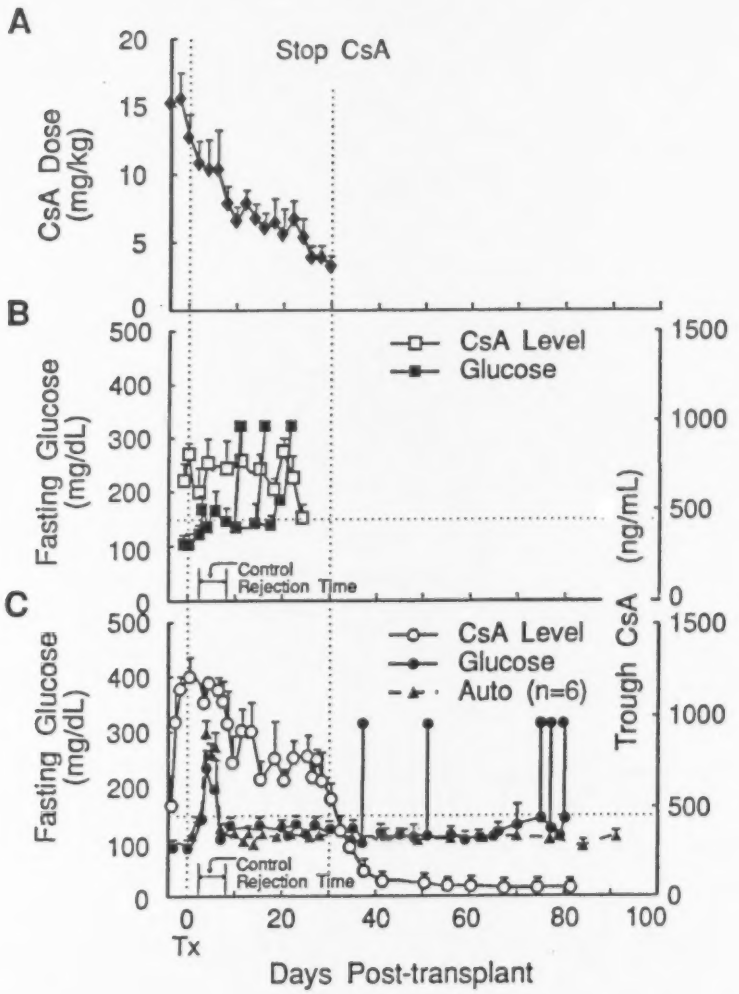

FIGURE 4-4: Function of purified single donor islets that were implanted into the omental pouch of eight dogs: a) daily CsA dosage; b) the graft function in three dogs that had low initial CsA levels; c) the graft function when high initial CsA levels were maintained in five dogs (versus recipients of intra-omental autografts without CsA). Failure of the allografts resulting in hyperglycemia was observed at 7, 21, 45, and 50 days after stopping CsA.

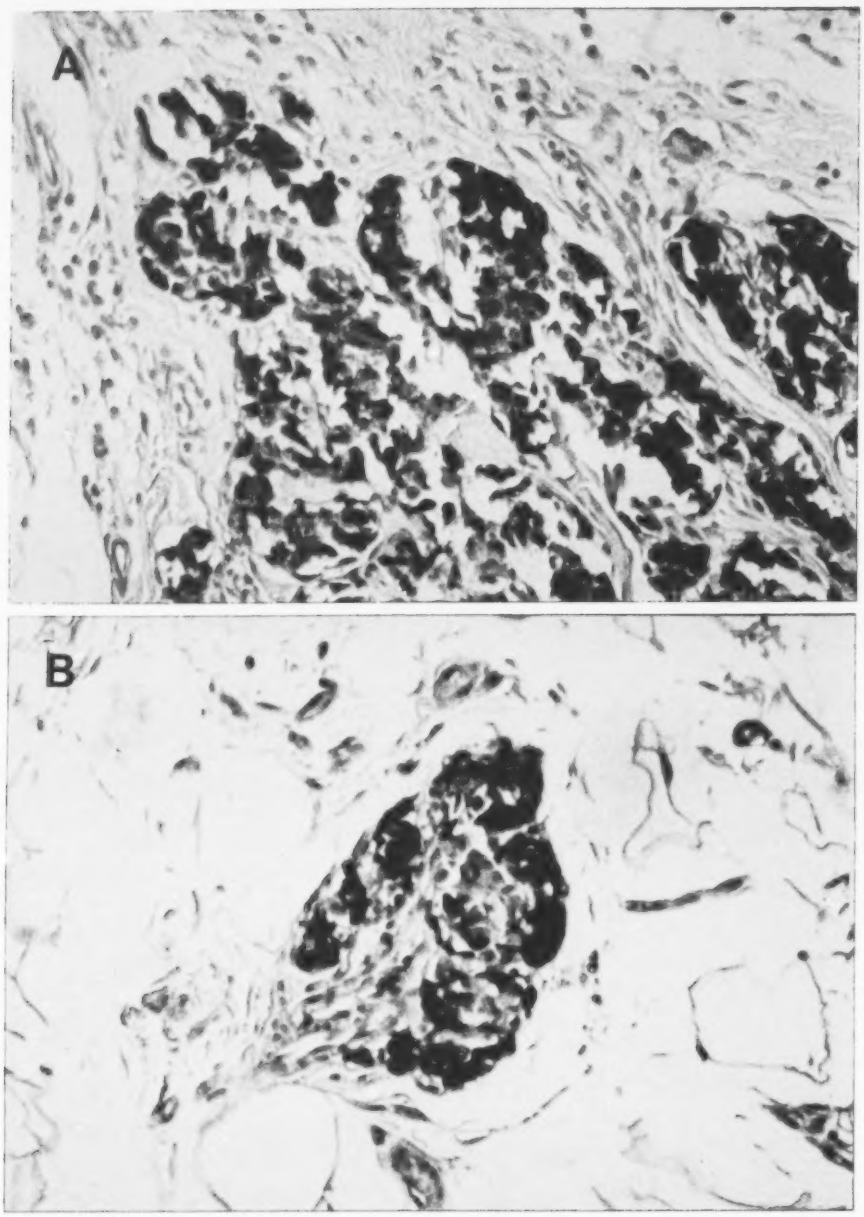

PLATE 3-1: Photomicrograph of intra-omental islet autograft 3 months after transplant. a) showing numerous well granulated islets localized between leaves of omentum; b) showing islet embedded within omental fat (immunocytochemical localization of insulin that is stained brown, original magnification 630X for both "a" and "b").

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

DISCUSSION

Mass isolation of purified viable islets, optimal transplant site, and effective anti-rejection therapy are three key factors for successful application of islet transplantation in treatment of IDDM. In this study we have used the dog as a preclinical model to optimize mass islet isolation protocols, to develop a new site for islet autografts and allografts.

Since Ricordi and coworkers (1) introduced the automated method for islet isolation, many centres have employed this method to improve mass isolation of islets in human and large animal models (1-5). However the efficacy of the automated method has not been well compared with the manual method. The data of the study demonstrates that, compared with manual method, combination of continuous collagenase perfusion and modified automated dissociation with gentle shaking could improve the yields and size distribution in canine islet isolation. Our major modifications after Ricordi's automated method include continuous collagenase perfusion which allows better enzyme delivery to the peri-insular matrix, gentle shaking technique to minimize mechanical trauma to the islets, and replacement of steel shaking chamber with glass chamber with which the dissociation procedure can be visually monitored.

Following collagenase digestion and mechanical dissociation, purification is another critical step for mass islet isolation. Compared with Dextran, Ficoll is more expensive as a purification medium and was found more toxic to the islets in small animal study (6-7). In this study we found the Dextran was as effective as Ficoll in canine islet purification. Both

Dextran and Ficoll purified islet autografts in the spleen could restore normoglycemia in pancreatectomized dogs. At in vitro perifusion both Dextran and Ficoll purified islets demonstrate biphasic insulin secretion responses, close to 3-fold of increased insulin release after glucose stimulation, and return to prestimulation level of insulin secretion when glucose challenge was discontinued. Data in this study cannot determine whether Dextran is less cytotoxic than Ficoll. However considering its inexpensiveness, and equivalent purification compared with Ficoll, Dextran may be a better purification medium for mass islet isolation in large animals. The efficacy of Dextran for human islet isolation needs to be investigated.

In this study we have devised an omental pouch site for canine islet transplantation. The threshold mass of islet autografts is 10 μL/kg in the omental pouch site, and 4 μL/kg in the splenic site. Normoglycemia was well maintained after islet autotransplant in both omental pouch and spleen groups. The metabolic function of intraomental islets was impaired compared with intrasplenic islets. The discrepancy of the threshold graft mass and metabolic performance between the two transplant sites may be due to that the islets in the spleen have better access to the blood supply, therefore are allowed better engraftment in the early stage of transplant.

The untreated islet allografts in the omental pouch site failed promptly with a mean survival time of 4.3±1.8 days. In contrast, the intraomental islet allografts had a prolonged survival with CsA therapy. In five dogs whose initial blood CsA exceeded 1000 ng/mL, normoglycemia was well maintained through 30 days CsA treatment and this persisted further for 34±9.5 days after cessation of CsA. The depth of initial immunosuppression appeared to be crucial to this success, since earlier graft failure were found in the dogs who had lower initial blood CsA level (<800 ng/mL). These requirements for CsA monotherapy are

consistent with findings in the studies of intrahepatic (8) and intrasplenic (9) islet allografts. The survival of intra-omental allografts after cessation of CsA compares favourably with survival of intrasplenic grafts previously reported from our laboratory (9). In the latter study, despite higher initial dosage of CsA, the mean survival time of grafts after cessation of CsA was only 11±2 days. Several factors may account for the different allograft survival time in the different sites. First, higher activities of cell-mediated immune effector responses in the spleen may accelerate the rejection of intrasplenic islet allografts. Second, lower clearance rate of lipophilic CsA from the fatty omentum tissue may maintain a higher local CsA concentration. Finally, presentation of donor antigen via lymphatics in the omental route may differ from direct injection in the spleen.

Although the present study does not specifically address the effects of CsA on islet function, this study did find adverse effects of CsA on the metabolic function of the islet allografts in the omental site. The PG level in CsA treated recipients was higher (128.0±1.0 mg/dL) with a reduced K-value at IVGTT when compared with autograft recipients whose mean fasting glucose level was 109.0±3 mg/dL. In CsA treated dogs, the K-value improved and PG level were found to decrease significantly to 113.0±1.0 mg/dL when CsA therapy was discontinued. These findings of an adverse effect of CsA are in agreement with those reported earlier for islets implanted into the liver (10).

The omental site offers several practical and theoretical advantages. The creation of the pouch is technically simple. Insulin and other hormones from the islets are secreted into the portal venous system. The omental tissue bearing islet grafts could be easily retrieved for biopsy purpose. The omental pouch site may facilitate local application of drugs, such as immunosuppressive agents (11) and capillary growth factor (12). Perhaps the most attractive advantage of this site is that the pouch may be suitable to localize mass quantities of microencapsulated islets in large animal and human.

CONCLUSIONS

1) Combination of continuous collagenase perfusion and automated dissociation with gentle shaking improves the yield and size distribution in canine islet isolation when compared to manual dissociation.

2) Intrasplenic autografts of islets isolated with manual or automated protocol can maintain normoglycemia in pancreatectomized dogs.

3) Dextran has the same efficacy as Ficoll in canine islet purification.

4) Dextran and Ficoll purified islets function in vivo and in vitro.

5) The threshold mass of canine islet autografts to reverse pancreatectomy induced diabetes is 10 μL/kg in the omental pouch site, and 4 μL/kg in the splenic site.

6) Metabolic efficiency of canine islet autografts in the omental pouch site is impaired compared with those in the splenic site.

7) Allografts of canine islets in the omental pouch site are rapidly rejected in untreated recipients.

8) Allografts of canine islets from an outbred single donor have prolonged survival in the omental pouch site with sufficient CsA immunosuppression (initial trough blood CsA level >1000 ng/mL).

9) Metabolic efficiency of CsA treated canine islet allografts is impaired compared with autografts. Cessation of CsA treatment may reverse the impaired metabolic functions.

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